ROLE OF AN EQUINE HOMOLOGUE OF GONADOTROPIN-INHIBITING HORMONE IN CONTROLLING SECRETION OF LUTEINIZING HORMONE IN THE MARE

A Thesis

by

LIGIA DIAS PREZOTTO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

May 2012

Major Subject: Physiology of Reproduction

ROLE OF AN EQUINE HOMOLOGUE OF GONADOTROPIN-INHIBITING HORMONE IN CONTROLLING SECRETION OF LUTEINIZING HORMONE IN THE MARE

A Thesis

by

LIGIA DIAS PREZOTTO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Co-Chairs of Committee, Gary L. Williams
Marcel Amstalden
Committee Member, Steven Brinsko
Head of Department, H. Russell Cross

May 2012

Major Subject: Physiology of Reproduction

ABSTRACT

Role of an Equine Homologue of Gonadotropin-Inhibiting Hormone in Controlling Secretion of Luteinizing Hormone in the Mare. (May 2012)

Ligia Dias Prezotto,

B.S., University of Sao Paulo, Pirassununga-SP-Brazil

Co-Chairs of Advisory Committee: Dr. Gary L. Williams
Dr. Marcel Amstalden

Four experiments were conducted to test the hypothesis that RF-amide related peptide 3 (RFRP3) negatively regulate the secretion of LH in mares. In Exp. 1, mares received native gonadotropin-releasing hormone (GnRH) continuously at a rate of 20 μg/h, delivered subcutaneously using Alzet osmotic pumps during the luteal phase of the estrous cycle. Mares were treated with i.v. bolus injections of 0, 500 and 1,000 μg eRFRP3 on days 4, 6 and 8 of cycle. Mean concentrations of LH in the peripheral circulation averaged 1.2±0.2 ng/mL and did not differ among groups before or following RFRP3 treatment. In Exp. 2, pituitary venous effluent was sampled for characterization of episodic release of LH. Mares received either saline or eRFRP3 (250 μg) i.v. every 10 min for 6 h beginning 2 h after onset of sampling. At hour 6, each mare was challenged with 1 mg GnRH. Neither mean ICS concentrations of LH (1.3 ± 0.2 ng/ml), nor frequency (3.6 ± 0.55 episodes/h), amplitude (0.2 ± 0.03 ng/ml), or duration (36.3 ± 3.5

min) of individual secretory episodes, differed between groups before or after eRFRP3 treatment. Area under the GnRH-induced LH curve (arbitrary units) also did not differ between control and RFRP3 treated mares (175.9 ± 11.4 vs. 192.6 ± 10.6). In Exp. 3, winter anovulatory mares (n=6) were treated continuously for 7 d with GnRH (100 μg/h) to stimulate synthesis of LH and increase circulating concentrations of LH to values similar to the breeding season. The ICS was catheterized for blood sampling and mares were treated with saline or RFRP3 (5 mg) in a replicated Latin square design. Treatment with RFRP3 failed to alter ICS mean concentration of LH (0.95 ± .03 ng/ml). Finally in Exp. 4, mares in the follicular phase of the estrous cycle were assigned randomly to receive either saline (n=3) or 10 μg/kg BW of oRFRP3 (n=3) in a single injection. No effect on mean concentration of LH was observed. In contrast to observations in birds and other mammals, results of the current experiments fail to provide evidence for functional activity of eRFRP3 or oRFRP3 in regulating LH release in the mare.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Gary L. Williams for the opportunity to join this program. His guidance, commitment, professionalism, support and dedication as an advisor were fundamental for the learning processes throughout my training. I sincerely thank you for constantly challenging and helping me to get this far.

I also want to express equal appreciation to Dr. Marcel Amstalden for all his patience, guidance, and help while I was in College Station. As well as a special thank you to Dr. Steven Brinsko, for serving as a committee member.

My gratitude is extended to the employees of the Texas AgriLife Research Station, Beeville, for their time and help while I lived there and conducted my experiments. Additionally, I would like to thank Mr. Lloyd Vaughn (Flying V Quarter Horses), Mrs. Patty Salge and Mr. Buddy Jones for their kindness in loaning the mares for these studies.

I would also like to acknowledge those who have been helping me to be whom I am and to get where I want to be: my parents, Jorge and Maria Auxiliadora Prezotto, and siblings, Laura and Lucas Prezotto for all the encouragement, support, motivation, inspiration and patience. In order words, for all the times you stood by me, for all the truth that you always made me see, for all the dreams you made come true, and for all the unconditional love that I always found in you even though you have being so far away from me.

Finally, I would like to acknowledge my fellow graduate students Andrea Lloyd, Ashley Brooke Keith, Brook Bradbury, Bruna Alves, and Michael Uzelac, and laboratory technician Sarah Sharpton for the friendship and help during my time in College Station. And, especially Rodolfo Cardoso and Jennifer Thorson for being my friends, collaborators, and support system during my entire time at Texas A&M.

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

The natural breeding season of the mare occurs during long days. Therefore, in the northern hemisphere, it is generally restricted to the interval between April and October. After this period, a majority of mares become anovulatory and ovaries remain quiescent throughout the winter. Most research in this area has focused on the roles of day-length [1], pineal melatonin [2], and gonadotropin releasing hormone (GnRH) secretion [3]. However, the neuroendocrine basis of equine reproductive seasonality has not been completely elucidated.

The use of artificial lighting to extend day-length and hasten onset of spring transition is well-documented. It is an effective managerial strategy used by many large farms; however, the method is not widely utilized by smaller breeders because of limitations in facilities [4].

Pineal melatonin release, which increases in response to decreased photoperiod, has long been implicated in the control of seasonal reproduction through potential effects on secretion of GnRH [2]. There is some evidence that melatonin reduces GnRH content of the hypothalamus in mares [5] but a direct effect of melatonin on hypothalamic secretion of GnRH has not been demonstrated. Therefore, this area of investigation requires much additional work.

This thesis was written in the format and style of *Domestic Animal Endocrinology*.

Gonadotropin-releasing hormone is the primary regulator of both LH and FSH synthesis and secretion [6, 7, 8], and existing dogma indicates that the winter anovulatory state in mares is a consequence of reduced secretion of GnRH. Therefore, some studies have focused on pulsatile [9, 10] and continuous [9, 11, 12] administration of GnRH to induce follicular growth and ovulation in mares during the anovulatory state. Although, such treatments have clearly demonstrated their ability to stimulate ovarian activity successfully, studies conducted in our laboratory have questioned whether seasonal anovulation is controlled only by limitations of endogenous GnRH secretion, or involves changes in pituitary responsiveness. Using the intercavernous sinus (ICS) cannulation technique to measure secretion of GnRH, Cooper reported no change in GnRH release during 4 different seasons [13]. This observation would support the hypothesis that winter anovulation in the mare is mediated by changes in gonadotrope responsiveness.

In 2000 Tsutsui et al. [14], reported the identification of a novel hypothalamic neuropeptide with a C-terminal LPLRF-amide sequence in the quail brain. This neuropeptide inhibited gonadotropin release in birds both *in vivo* and in cultured anterior pituitary cells. The functional RF-amide was named, gonadotropin-inhibiting hormone (GnIH). Although, other neuropeptide (e.g. endogenous opioid peptide (EOP) are known to project to the median eminence (ME) and to the posterior pituitary, and inhibit LH release by direct actions in the pituitary [15, 16], their physiological relevance regarding the control of gonadotropin release is controversial. Thus, GnIH became the most

physiologically relevant hypothalamic peptide shown to inhibit gonadotropin release in vertebrates and has been widely studied in this regard.

Following its discovery in the avian brain, studies were conducted in other species, such as mammals, amphibians, and fish [17, 18, 19, 20, 21, 22]. These studies resulted in the finding of similar GnIH-related peptides broadly termed RF-amide related peptides (RFRP). All of these RFRP possess a LPXRF-amide sequence in the C-terminus, similar to that observed in the avian GnIH [19, 23, 24, 25, 26, 27]. The RFRP gene encodes more than one peptide (i.e. RFRP1, RFRP2, and RFRP3). However, the RFRP3 appears to have the most potent effects on secretion of LH [19, 28].

Initial work in birds focused on the possibility that the main effects of GnIH were at the hypothalamic level through effects on GnRH neurons [14, 17, 29, 30, 31]. However, studies in sheep were not able to confirm a decrease in GnRH concentrations in portal blood plasma after GnIH administration. Whereas, some investigators were able to report effects on GnRH expression in hypothalamic cells after giving RFRP3 through intracerebroventricular (ICV) injections [32], *in vivo* studies mainly measured dose-related reductions in secretion of LH after injection of GnIH/RFRP3 [18, 20, 29, 33, 34, 35].

The potential role of RFRP3, the mammalian homolog of avian GnIH, in equine reproductive seasonality has not been elucidated. Because of the economic importance of the horse and its profound seasonality, it is essential that these studies be undertaken. The objectives of the studies described herein were to determine 1) whether eRFRP3 have the ability to decrease mean concentrations of LH in peripheral plasma of mares in

the luteal phase of the estrous cycle during concomitant infusion of GnRH, 2) if continuous infusion of eRFRP3 has the ability to suppress the pulsatile release of LH in the intercavernous sinus (ICS) of mares during the follicular phase, 3) if eRFRP3 injected into anovulatory mares treated continuously with GnRH (i.e. synthesis and release of LH is similar to what is observed during breeding season) results in a suppression of LH secretion, and 4) if ovine oRFRP3 injected into mares during the follicular phase suppresses LH secretion in the ICS. The overal hypothesis is that either eRFRP3 or oRFRP3 will disrupt the normal pattern of LH secretion as detected in the peripheral circulation or in the ICS effluent.

CHAPTER II

REVIEW OF LITERATURE

2.1 Overview of reproduction in the mare

Mares are seasonal long-day breeders with a natural breeding season extending on average from April to October in the Northern Hemisphere. Approximately 85% of mares become anovulatory during the late fall and winter. Resumption of ovarian cycles can occur as early as February or March and as late as May or June [1]. Around the time of the autumn equinox a reduction in synthesis and release of anterior pituitary luteinizing hormone (LH) occurs, followed by cessation of ovulatory cycles in most mares [36].

Continuous infusions of GnRH into mares from mid-January or later can effectively induce secretion of LH and follicle development in winter anovulatory mares [11, 37]. These results imply that a reduction in secretion of GnRH is the likely limiting factor during seasonal anovulation. In addition, the ability of pituitary gonadotropes to respond to GnRH changes as the winter anovulatory period progresses, with a lesser response in late February compared to late December [11, 38]. However, it is not known whether this occurs as a result of innate changes in gonadotrope responsiveness to GnRH or due to chronic inactivity of cellular machinery following an extended period of limited exposure to GnRH.

The measurement of pulsatile release of both GnRH and LH in the mare can be made using the technique of ICS cannulation developed by Irvine [39], which allows

sampling from the anterior pituitary draining vein effluent. More recently, the technique has been used by Cooper et al. [13] and Velez et al. [11] in our laboratory. In the former case, it was observed that the marked decline in secretion of LH in winter was not accompanied by similar declines in ICS concentrations or pulsatile release of GnRH [13]. However, these data remain controversial and additional critical research steps are necessary in order to confirm their accuracy.

2.2 Potential roles for seasonality in reproduction of the mare

2.2.1 Natural and artificial photoperiods

It is well accepted that photoperiod is a major regulator of seasonality in horses and, over the years, investigations have focused on its different physiological effects. Regardless of whether lighting conditions are considered inhibitory or stimulatory, mares exposed to constant lighting conditions become refractory to that signal and their circannual reproductive rhythms resume [40, 41, 42]. This can be overcome by exposing the animal to periods of light and dark at regular intervals. Mares exposed to artificial lighting that extends day-length to 15-16 h during the winter and early spring exhibit an accelerated onset of the breeding season, which reinforces the influence and advantage of artificial lighting [43]. However, mares exposed continuously to 16 h of light and 8 h of darkness eventually return to anestrus [40, 41, 42].

Several early studies utilized different combinations of light and dark periods with the objective of determining which would induce ovulation in anovulatory mares [41, 44, 45, 46, 47]. When intact anovulatory mares were exposed to increasing photoperiod using artificial lighting, the first ovulation occurred earlier [48]. The

approaches used different combinations of light and dark periods in an effort to optimize the hastening of ovulation [40, 41, 42, 43, 44, 45, 46, 47]. However, variations in the circadian rhythms of animals in experimental conditions mimicking the perception of sunrise and sunset, which is important for the placement of the photosensitive phase, are different to those compared to natural conditions. Thus, it can be concluded that it is not possible to completely duplicate this situation under experimental conditions [49].

2.2.2 Importance of nutrition

The influence of body condition score on the average interval to first ovulation of the breeding season [50] becomes apparent when mares are supplemented with high concentrate diets. Mares ovulated earlier in the spring transition under this regimen and when BW gain was promoted during the early spring [51, 52]. The combination of diet supplementation and artificially extended photoperiod also positively influence the onset of reproductive activity in the spring [53]. Moreover, mares in low body condition exposed to a high-dietary energy intake and improving body condition during spring transition had a shortened interval to first ovulation [54]. Mares grazing on pasture with grass of high quality exhibit a similar phenomenon [55].

2.2.3 Role of melatonin

Melatonin is a key hormone controlling seasonal reproduction in mammals. Studies demonstrating that the pineal gland translates photoperiodic signals received by the eyes to other endocrine effects through changes in melatonin secretion were important for our current understanding of mechanisms regulating seasonal reproduction in horses and other mammals [56, 57, 58]. Production of melatonin from pineal explants

collected throughout the year is greater in December and January and returns to lower, breeding season values by February [43, 57, 59]. This observation corresponds to similar changes in concentrations of hydroxyl-indole-o methyl transferase (HIOMT), the enzyme involved in the synthesis of melatonin in the pineal [59]. The pattern and duration of melatonin secretion can be changed when mares are exposed to artificial light during the natural dark phase [44, 57].

Mares which were pinealectomized and exposed to an extended photoperiod did not exhibit hastening of reproduction transition [60], and treatment with melatonin results in a similar effect [44, 57]. In addition, mares treated with melatonin implants did not exhibit any ovarian effects when implanted on the shortest day of the year, but onset of the breeding season was advanced when treatments were applied close to the summer solstice [58]. Exogenous melatonin has been shown to affect hypothalamic concentrations of GnRH when injected in ovariectomized mares during breeding season and in sheep [61, 62]. However, conclusions drawn from such studies appear to be in conflict with others in which melatonin seemed to affect circannual rhythms rather than reproductive activity directly [49].

2.2.4 Patterns of LH and GnRH secretion

Several studies have provided evidence that the photoperiodic regulation of equine seasonal reproduction is steroid-independent [63, 64]. The secretion of LH in intact and ovariectomized mares has been shown to follow a seasonal pattern, with increased secretion during the breeding season and decreased secretion during the anovulatory period [65, 66, 67, 68]. However, there does not appear to be a similar

pattern of change for follicle stimulating hormone (FSH) [67]; although, minor changes have been observed [38]. Mares injected with GnRH during the transition period from the anovulatory phase into the breeding season had release of LH and FSH stimulated [69].

During the spring transition period, before first ovulation of the breeding season, mares often exhibit prolonged periods of estrous behavior in the absence of significant ovarian activity. However, mean circulating concentrations of LH remain relatively low until 6-7 days before the first ovulation [66, 67].

A reduction in hypothalamic content of GnRH in anovulatory mares is correlated with low stores of LH in the anterior pituitary during late winter [70]. However, the restoration in GnRH concentrations has been shown to occur earlier in the spring than restoration of pituitary LH. Maximal adenohypophyseal concentrations of LH are not observed, until mid-breeding season [70, 71]. Additionally, there appear to be differences in hypothalamic distribution of GnRH immunoreactivity during the breeding and non-breeding seasons and, in spite of findings in our own laboratory, there is evidence that the release of GnRH during the anovulatory season is reduced [71]. In the horse and some other species, LH response to exogenous GnRH is used to estimate pituitary responsiveness to endogenous GnRH. Mares in different reproductive states received different doses of GnRH which induced pulsatile LH secretion comparable to what is observed endogenously, implying that the differences observed in concentration of LH between seasons is due to a difference in endogenous GnRH secretion [72]. Moreover, a difference in overall mean secretory rate of GnRH among groups in their

breeding and non-breeding seasons was observed using the push-pull perfusion technic [73].

In sheep, estradiol plays a definitive role in reproductive seasonality. During the non-breeding season, there is an increase in the negative feedback sensitivity to estradiol through activation of the dopaminergic system and consequent suppression of the pulsatile release of GnRH and LH [74]. To the contrary, when mares were treated chronically with estradiol during nonbreeding season, mean concentrations of LH increased [64]. This indicates that winter anestrous is independent of estradiol negative feedback in the mare.

2.2.5 Thyroid hormone

Thyroid hormones have been demonstrated to contribute to the regulation of seasonal breeding in some species, such as sheep [75]. Thyroidectomy abolishes the reduction in episodic release of LH that occurs in intact ewes at the end of the breeding season. However, secretion of LH was similar in intact and thyroidectomized ewes during the breeding season [75]. The requirement for thyroid hormones occurs only during a small window of time at the end of the breeding season in ewes [76]. The secretion of LH can be inhibited in ewes thyroidectomized late in the breeding season and then treated centrally with thyroxine (T4) [77]. The role of thyroid hormones in the mare has not been extensively studied. Circulating concentrations of thyroid hormones in horses are greatest during the winter compared to other seasons [78]. Unlike sheep [77], thyroidectomy does not alter timing of the onset of the anovulatory season in mares.

2.3 Circannual clock mechanisms

The study of circannual clock mechanisms is an recognized concept based on a working hypothesis involving the existence of adult stem cells in the brain, pituitary gland, and some peripheral tissues that appear to undergo synchronized division [79]. These cells proliferate, migrate and differentiate, providing the necessary substrate for specific physiological changes over time, and have cell death as a trigger for the beginning of the following cycle. This model has been characterized in species such as primates including human [80, 81], and in several hibernating [82, 83, 84, 85, 86, 87], seasonal breeding [88, 89] and seasonal migratory [90, 91, 92] species.

No changes in the circannual rhythm have been observed when seasonal animals (birds, mammals, and insects) were maintained in a constant light and dark cycle that are different from the natural exposure to a 24 h day (e.g. 23 or 25 h d) [90, 93, 94, 95]. The ability of the nervous system to monitor photoperiodic time enables the system to detect the annual cycle of day-length and synchronizes physiological changes throughout seasons which are dependent on the endogenous circadian system. When photoperiod changes it is possible to observe activation or inhibition of seasonal responses, or alteration of endogenous circannual rhythm generators that synchronize with the environment in a long term fashion [96, 97, 98].

The suprachiasmatic nucleus (SCN) serves as a pacemaker timing the circannual rhythm including activity and sleep, body temperature, pituitary activity, among other diurnal and nocturnal rhythmicity involving melatonin activity. This occurs by influencing the timing of duration of the melatonin rhythm. This also influences the

reading of melatonin signals by the pars tuberalis (PT), one of the more well-studied targets in which circadian clock genes have been characterized [99]. The location of the PT, and the fact that it is a melatonin target tissue with a high density of melatonin receptors, makes it a specialized site for regulating the circannual clock [100, 101]. It appears to function as a regulator of long-term cycles in several physiological systems. For example, some specialized PT cells (PT thyrotropes) [102] are responsible for TSH secretion, which acts locally in the pituitary gland and regulates thyroid hormone dependent mechanisms [97, 103]. In mammals, information sent from the retina to the SCN coordinating the production of melatonin by the pineal gland in a 24 h period, is the mechanism coding photoperiod [90, 104].

The endogenous circannual rhythm, driven by specific genes, drives seasonal reproductive cycles in sheep, and these cycles are maintained even under constant photoperiod [105]. This phenomenon is termed the photorefractory response. Procedures such as pinealectomy or denervation of the pineal through superior cervical ganglionectomy can be utilized to dissociate the circannual and circadian timing systems. The operations remove the melatonin signal and thus block physiological responses to photoperiod [98, 106]. Specific clock genes controlling secretion of GnRH in the hypothalamus, unlike those in the PT, have not been characterized.

One of the limiting factors for the study of the circannual clock is length of time required to observe changes within or between fractions of a circannual cycle compared to those needed for typical neuroendocrine feedback loops [107]. The minimal time for observing these changes is dependent on cyclical tissue regeneration. The theory

underlying this process has been termed the “histogenesis hypothesis” [79]. Photoperiod and other environmental cues are responsible for modulating these regenerative cycles, and may do so by suppressing or prolonging the proliferative phase of new cells. These processes are analogous to observed changes in response to daily light signals that affect the rhythmic expression of clock genes [108].

The histogenesis hypothesis considers regenerative mechanisms in the brain, pituitary, and in peripheral body organs that are involved in the control of circannual rhythms. The model shows that all tissues have certain autonomy in regenerating their own cells and contributing to long-term timing of some physiological cycles [79]. In sheep, this system is synchronized by the PT and mediobasal hypothalamus, and coordinated by the SCN which controls the functional state of the whole body through circannual pacemakers [79].

2.4 The role of endogenous opioid peptides

Endogenous opioid peptides (EOP) were the first to be classified and identified as hypothalamic inhibitory peptidergic signals regulating pituitary secretion of LH [109, 110, 111]. They have been divided into three classes of biological activity according to the hypothalamic regions in which they were found [112, 113,114, 115, 116]. However, all of these classes overlap in regions that are innervated by GnRH neurons and have shown the ability to inhibit secretion of LH when studied in ovariectomized rats [110, 111,117]. It has also been proposed that the EOP system may influence the neural clock and its ability to govern GnRH surges in rats [118]. Numerous studies have examined

the influence of EOP on the hypothalamic secretion of GnRH and its subsequent effects on gonadotropin release [119, 120, 121].

The EOP antagonist naloxone has positive effects on secretion of LH in several species, including in mares during the luteal phase [122] and anovulatory season [121], and in the human [123], sheep [124, 125, 126], and pig [127]. A relationship between opioid inhibition and progesterone has been observed in cyclic mares [122, 128] as well as other species [123, 127, 125, 126]. Other investigators have shown that the release of opioids is independent of ovarian steroids [121], and in some animal models (e.g., anovulatory mature sheep) naloxone failed to have any effect on release of LH [124, 129].

2.5 Potential role for GnIH/RFRP3 in reproductive seasonality

2.5.1 Localization and peptide sequence

One of the more recent developments in reproductive endocrinology has been the discovery of new family of hypothalamic signaling peptides, the RF-amide related peptide (RFRP). RF-related peptide 3(RFRP3), a peptide capable of suppressing the secretion of LH in birds and subsequently named gonadotropin-inhibiting hormone (GnIH), was the first RFRP identified and described by Tsutsui et al. in 2000 [14]. A number of studies have indicated that GnIH may play an important role in the seasonal regulation of reproduction of avian species [14, 17, 24, 29, 30, 130, 131] through its ability to control secretion of LH.

Immunohistochemical evidence of this peptide has been demonstrated in the hypothalamus [23]. Investigations in the brains of mammals, amphibians, and fish

followed these initial reports [17, 18, 19, 20, 21, 22]. As a result, 3 RF-amide related peptides (RFRP1, 2 and 3), with RFRP3 being the homolog of GnIH, and GnIH/RFRP3 receptors (GPR147, OT7T022, and GPR74) [19] have been observed and characterized in numerous species [17, 18, 19, 20, 21, 22, 24, 25, 26, 130, 132, 133, 134].

In our laboratory, we have demonstrated that RFRP3-immunoreactive neurons (Fig. 1A) are located in the dorsomedial hypothalamus, similar to that reported previously in other mammalian species [17, 18, 19, 20, 135]. It was also observed that RFRP3-immunoreactive neuronal fibers are in close proximity to GnRH neuronal cell bodies and dendrites (Fig. 1B), supporting a potential role for synaptic regulation of GnRH neurons by RFRP3. The presence of RFRP3-immunoreactivity was also observed in the infundibulum, a classical site for the release of neurohormones (releasing and inhibiting hormones) into the hypothalamic-hypophyseal portal vasculature for regulating adenohypophyseal function. Using the equine genomic sequence homologous to avian GnIH, an equine-specific primer was designed for the equine RFRP and a partial cDNA sequence of approximately 420 bp was amplified by PCR as shown in Fig. 2 [Amstalden et al unpublished data].

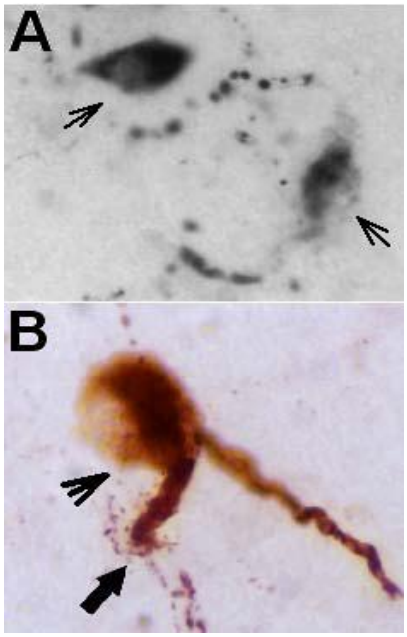

Fig. 1.(A) GnIH-immunoreactive neurons in the dorsomedial nucleus of the equine hypothalamus (arrows). (B) Double-label immunocytochemistry for GnRH (brown) and GnIH (purple) in equine hypothalamic sections. The close proximity of GnIH containing fibers to GnRH dendrites (arrow) and the cell body (arrow head) can be observed in the photomicrograph [Amstalden, Bentley and Williams unpublished observations].

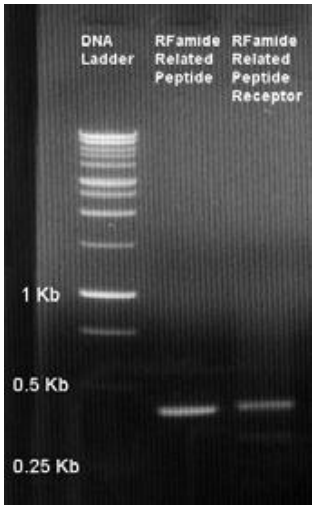

Fig. 2. Amplification of RFRP3 and RFRP3 receptor from equine hypothalamic cDNA. PRC products of approximately 420 BP were generated using primers specific for equine sequences [Amstalden, Bentley, and Williams unpublished observations].

When GnIH was first characterized in quail, most of the GnIH immunoreactive fibers were observed in the median eminence (ME) of the hypothalamus, and in the dorsal motor nucleus of the vagus in the medulla oblongata [136]. Moreover, when investigated in sheep, RFRP3-containing hypothalamic neuronal fibers were shown to project extensively into the neurosecretory zone of the ME [18]. Additionally, in hamsters it could be observed that these fibers were scattered in the inner layer of the same area [20].

The structure of RFRP peptides always possesses a LPXRF-amide sequence, where X represents L or Q with a C-terminal in common. The horse sequences are similar to the ones observed in humans, and sheep, containing the predicted pre-protein with RF-amide related peptides, and one RFRP-like sequence [17, 18, 20]. However, the mammalian homologue differs in amino acid sequence from that of avian [19, 23, 24, 25, 26, 27]. Therefore, the GnIH-related peptide discussed herein will be referred to as RFRP3 until full characterization of their physiological function is determined in mammalian species.

2.5.2 GnIH/RFRP3 effects on GnRH and LH release

Having confirmed the presence of GnIH and its homologs in several species [14, 18, 20, 30, 137], additional research was undertaken to understand their roles in reproduction and other physiological processes. Peptides related to the GnIH/RFRP family originate in the paraventricular nucleus (PVN), which appears to be the only source of GnIH production. In several bird species, some fiber terminals extend to the external layer of the ME where they play a role in controlling pituitary gonadotropin

secretion [17, 29, 30, 31, 131, 138]. However, in Syrian hamsters GnIH fibers have been observed primarily in the internal rather than external layer of the ME [20]. This observation indicates that GnIH might not be able to reach the necessary vessels to regulate pituitary secretion [17, 20]. GnIH immunoreactivity exhibits marked changes across seasons in seasonal breeding birds and rodents [17, 139].

The role of GnIH/RFRP3 in regulating GnRH release has been demonstrated by GnIH neurons making axosomatic contact with GnRH neurons, putatively decreasing the release of GnRH release in avian and mammalian species [31, 134, 139]. When tissue from the preoptic area of male rats was double-labeled and stained for GnRH and RFRP3, a mean of 75% of GnRH cell bodies were shown to be in contact with RFRP3 fibers [24]. Moreover, in mice RFRP3 appears to directly inhibit the firing rate of a proportion of GnRH cells [140].

Despite evidence that GnIH/RFRP3 may directly affect function of GnRH neurons, additional observations have indicated expression of the peptide's receptor on gonadotropes and direct effects on secretion of LH at the adenohypophyseal level [31, 33, 138]. In order to explain how RFRP3 may affect pituitary gonadotropes, it is instructive to understand the mechanism of GnRH-induced release of LH. Following GnRH receptor binding there is an increase in intracellular inositol triphosphate [141, 142], which promotes Ca^{2+} discharge from intracellular Ca^{2+} through opening of calcium channels [143]. With the increase in cytoplasmic calcium influx, LH release is stimulated [141, 142]. Clarke et al [18] has shown that when sheep pituitary gland cell

cultures are treated with RFRP3, the generation of the intracellular free calcium required for LH release is blocked.

Additional studies have demonstrated that GnIH/RFRP3 reduces LH release even when its release is stimulated by exogenous GnRH [35, 144]. After continuous infusion of different doses of GnIH via osmotic pumps for 2 wk in mature male quail, a decrease in LH concentration in the plasma from peripheral blood was observed [145]. Furthermore, inhibition of LH secretion has been shown in pituitary cell cultures of male quail [29], rat [35], chicken [146], and sheep [18], when treated with RFRP3. After infusing RFRP3/GnIH in hamsters [17, 20], and in rats [34, 35, 147], centrally or peripherally, serum concentrations of LH also decrease. In addition, castrated and photostimulated, white-crowned sparrows treated with quail GnIH showed a rapid decrease of LH concentration in plasma [131]. Because the quail sequence was effective in song sparrows and white-crowned sparrows [131], it seems that GnIH is heterospecific. When injected intravenously (i.v.) in ovariectomized [35, 144], and intact rats [137], in ewes [14], and in castrated male calves [33], RFRP3 suppresses the pulsatile release of LH and mean concentration of LH. All these results reinforced the suggestion that the RFRP3 may have its main action at the pituitary level.

2.5.3 Dose response relationship for GnIH/RFRP3

Among experiments indicating the presence, localization, and effects of GnIH/RFRP3, several have focused on dose relationships *in vivo* and *in vitro* in different species. In sheep pituitary cell cultures, RFRP3 appears to have effects at picomolar

concentrations [18]. This is somewhat anomalous to most other physiologically-active peptides which generally act in the nanomolar range [e.g., kisspeptin; 148].

When given as i.v. injections, RFRP3/GnIH has been shown to be effective in suppressing either circulating concentrations of LH or its pulsatile release at a dose of 1 μg in rats [35]. In sheep, a loading dose of 50 μg followed by a continuous infusion of 200 μg for over 1 h was effective in suppressing LH release [18]. Effective doses of 600 ng in Syrian hamsters [20], 1,000 ng in male song sparrows [131], and 90 μg injected every 10 min for 1 h in male calves has been observed [33]. Furthermore, intracerebroventricular (i.c.v.) injections of 0, 100, 300, or 500 ng were tested in ovariectomized Syrian hamsters [20]. However, only the concentration of 500ng was observed to be effective in this species.

2.5.4 Potential role of RFRP3 in feeding behavior

The RF-amide family of peptides may also play roles in the control of feeding behavior. Whether such effects will prove to be as important as those involving reproduction [137, 145, 147, 149, 150], prolactin release [32, 151, 152], or blood pressure [153] (effects that have been related to the entire RF-amide family) remains to be determined. However, studies in a wide range of species, including mammals, have shown that at least 3 of 5 known mammalian genes encoding for peptides of the RF-amide family have cognate receptors that are compatible with a physiological role in feeding behavior [154]. In rats and chicks treated with RFRP3, food intake was significantly increased [137, 155].

2.5.5 Melatonin effects on GnIH/RFRP3

Melatonin plays an important role in reproduction of seasonal-breeding animals through annual changes in pineal physiology [2, 156, 157, 158, 159, 160]. However, a functional link between melatonin secretion and GnIH [56, 161, 162, 163] has not been fully described. When melatonin concentrations were experimentally decreased in quail a concomitant decrease in the expression of GnIH precursor mRNA in the diencephalon was observed [130]. Therefore, it seems that GnIH expression is induced by a direct action of melatonin on GnIH neurons via its receptor. Moreover, the role of melatonin in the regulation of GnIH release and its correlation with LH release in quail has also been investigated. Administration of melatonin in varying doses to hypothalamic explants resulted in an increase in GnIH release, particularly in explants collected from animals exposed to long day photoperiods and incubated in dark conditions [130]. In addition, experiments in hamsters indicate that the expression of RFRP3 genes may be controlled by melatonin [164].

CHAPTER III

EFFECTS OF MAMMALIAN HOMOLOGUES OF GONADOTROPIN-INHIBITING HORMONE ON SECRETION OF LH IN THE MARE

3.1 Introduction

Reproductive seasonality in the equine species has been studied for several decades. However, the changes that occur in the hypothalamic-hypophyseal axis to regulate the different secretion patterns of gonadotropic hormones, particularly LH, observed during the breeding season and the anovulatory period have not been completely elucidated [11].

Methods to achieve an earlier spring transition by manipulating photoperiod with artificial lighting have been successful and adopted by the equine breeding industry [1]. The basis of this effect is through neural impulses traveling from photoreceptors in the eye through the suprachiasmatic nucleus (SCN) and superior cervical ganglion (SCG) to the pineal gland [104,165]. The latter process regulates pineal synthesis and secretion of melatonin which influences hypothalamic secretion of GnRH through currently undefined mechanisms.

A direct effect of melatonin on secretion of GnRH, which drives synthesis and secretion of both LH and FSH has not been demonstrated [2, 5]. Nonetheless, studies reported by Irvine and Alexander [72], utilizing the intercavernous sinus cannulation (ICS) technique, as well as others using push-pull perfusion [73], have concluded that the secretion of GnRH is decreased during the winter anovulatory period. Work in our

laboratory using the ICS technique, which sampled mares throughout the four seasons, has failed to confirm those findings [13]. Nonetheless, when seasonal anovulatory mares are infused hourly [9, 166] or continuously [37, 38] with native GnRH, anterior pituitary secretion of LH is dramatically increased, follicular development follows, and spontaneous ovulation occurs at a high frequency [11, 37, 38]. Therefore, speculations as to whether GnRH is limiting in winter or whether the gonadotrope becomes more resistant to an uninterrupted, endogenous secretion of GnRH in the horse have persisted.

The RF-amide family of peptides (RF-related peptides 1, 2, and 3) has been shown to regulate various physiological functions, including food intake and reproduction [34, 137, 145, 147, 149, 150, 154, 155]. The functionality and regulation of reproduction by one of these peptides, RFRP3, was first characterized and investigated in the quail. In avian species RFRP3 has been named gonadotropin-inhibiting hormone (GnIH) due to its suppressive effects on the release of LH [14]. Moreover, GnIH has been proposed to function as a major regulator of reproductive seasonality in seasonally-breeding birds [14, 131], and could play a similar role in seasonally-breeding mammals [18, 20]. In some mammalian species, GnIH and its mammalian homologue (RFRP3) have been shown to suppress the secretion of LH and adenohypophyseal responsiveness to GnRH [18, 19, 20] in a dose-dependent manner. Thus, if RFRP3 regulates seasonality in mammals, it may do so by direct effects at the anterior pituitary. As a first step in testing this hypothesis in mares, studies presented herein determined whether the predicted sequence for equine RFRP3 (eRFRP3), or the sheep homologue of GnIH

(oRFRP3), could disrupt the endogenous secretion and GnRH-induced release of LH in mares during different physiological conditions.

3.2 Materials and methods

All experiments had animal-related procedures approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System.

3.2.1 Experiment 1

Preliminary Study: Equine RFRP3 (eRFP3) effects on peripheral plasma LH in GnRH-treated mares during the luteal phase.

3.2.1.1 Hypothesis

Administration of eRFRP3 suppresses mean concentrations of LH during continuous infusion of GnRH.

3.2.1.2 Specific objectives

Determine whether eRFRP3 effectively suppresses mean peripheral concentrations of LH of mares in the luteal phase while treated continuously with native GnRH (20μg/h) to create an LH secretion pattern similar to that observed during the follicular phase [11].

3.2.1.3 Animals and study location

Six Quarter Horse mares ranging from 350-590 kg body weight and 2-19 years of age provided by local breeders were maintained on pasture (Coastal Bermuda grass) at Texas AgriLife Research-Beeville and supplemented with a mixed grain concentrate (12% crude protein; Falls City Milling, Falls City, TX) to maintain body condition score

between 5 and 6 on a 1 to 9 scale. Mares were teased daily with a stallion, and the day of ovulation was determined by transrectal ultrasonography. Three days after ovulation, mares received a subcutaneous osmotic pump (Alzet 2ML1; Durect Corp., Cupertino, CA) delivering native GnRH (20 µg/h) diluted in saline continuously for 7 d (Fig. 3). A jugular catheter was also inserted at this time and sutured to the neck using aseptic technique. Mares were assigned in a replicated Latin square design to receive saline, 500 or 1000µg of eRFRP3 (Table 1).

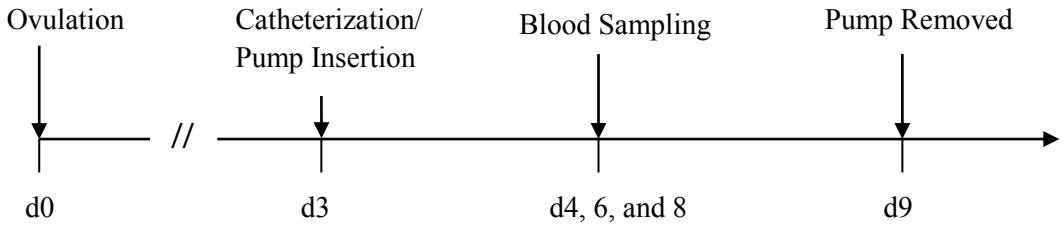

Fig. 3. Time line for experiment relative to ovulation day.

Table 1: Schedule relative to RFRP3 treatment and sampling.

Mares	Day of Luteal Phase					
	4		6		8	
1 and 6	A1	A6	B1	B6	C1	C6
5 and 4	B5	B4	C5	C4	A5	A4
3 and 2	C3	C2	A3	A2	B3	B2

Treatments : A= saline; B=500µg of RFRP3; C= 1000µg of RFRP3.

On the day of sampling, mares were tied loosely and provided water and hay during the sampling procedure. Jugular blood (12 mL) samples were collected every 15

min for 4 h for determination of mean concentrations of LH by radioimmunoassay (RIA). Mares were heparinized (40,000 IU sodium heparin, i.v.) daily to prevent clot formation in the jugular catheter. The catheter was also flushed with 2.0 mL (void volume of cannula and extension) of a heparinized solution containing 300 IU/mL following each collected sample. Samples were placed into tubes containing 50µL of a 5% EDTA-heparin solution (10,000 IU/mL) to prevent coagulation and were kept on ice until centrifugation for plasma collection after harvesting. Plasma was stored at -20º C until hormone analysis. Jugular cannulation and pump insertion were performed as described in *3.2.1.4*.

3.2.1.4 Alzet osmotic pump insertion and jugular catheterization

Two days after ovulation was observed (day 3 of the luteal phase), the mare was placed in a stock and an area above to the point of the shoulder measuring approximately 5 x 5 cm was clipped, scrubbed with an iodophore, and disinfected with tame iodine solution. Hands were scrubbed and sterile surgeon's gloves were worn. Anesthesia was performed with a solution of Lidocaine HCl (2%; 3-5 mL), Vedco, Inc, St Joseph, MO, injected subcutaneously). A 1.5-cm incision was made through the skin with a sterile scalpel. A pocket was created under the skin with a sterile blunt instrument to accommodate the Alzet osmotic pump. On the jugular groove an area measuring approximately 2 x 4 cm was clipped and disinfected as described above. A 12-gauge needle was inserted in the jugular vein and a polyethylene catheter (Becton Dickinson and Company, Sparks, MD) was inserted through the needle and sutured to the skin after the needle was removed. To close both incisions a non-absorbable (encased nylon

polyamide (RXVeterinary, Grapevine, TX)) suture (No. 2) was used. The areas were dressed with an approved wound dressing for horses. After the completion of the sampling schedule described in *3.2.1.3* the i.v. catheter and the pump were removed through the re-opening of the same incision, which were closed again with the non-absorbable suture. Suture was removed after 7 d.

3.2.1.5 eRFRP3 sequence synthesis and doses

Using primers for the eRFRP gene, a partial cDNA sequence of approximately 420 bp was amplified by PCR and cloned into a vector. The predicted sequence of the eRFRP3 has been determined to be Ile-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH2. The peptide was synthesized by-AUSPEP Clinical Peptides (Tullamarine Victoria, Australia) and used for this and the subsequent studies. Peptide validation was performed by high-performance liquid chromatography and mass spectral analysis (98% of purity and 983 of molecular weight). Doses used in this experiment were determined by extrapolating, based on BW, from doses used previously in other species such as sheep [18] and cattle [33]. The peptide was delivered in a bolus i.v. injection at the onset of sample period.

3.2.1.6 Hormone analysis

Concentrations of LH in plasma of all samples were determined by double antibody RIA validated previously in this laboratory [37]. For preparation of iodinated (I^{125}) tracer and standard (0.1, 0.2, 0.4, 0.8, 1.0, 2.5, 5.0, 10.0, and 20.0 ng/mL), a highly purified equine LH (eLH AFP-5130A) was used. A specific equine LH antiserum (AFP-240580Rb) at a dilution of 1:125,000 yielding an average binding (B/B0) of ~ 32.5%

was employed. Minimum detectable concentration was 0.1 ng/mL with mean intra- and inter-assay CV of 6.4 % (± 0.8) and 6.4%, respectively.

3.2.1.7 Statistical analysis

The effect of treatments on concentrations of LH was analyzed utilizing the GLM procedure of the Statistical Analysis System (SAS Inst., Inc., Cary, NC). The statistical model tested the effect of RFRP3 dose on mean concentrations of LH, using mare as the subject and time as the repeated variable.

3.2.2 Experiment 2

Pattern of LH secretion in pituitary venous effluent of the intercavernous sinus (ICS) of follicular phase mares treated chronically with eRFRP3 followed by a GnRH challenge.

3.2.2.1 Hypothesis

Chronic administration of eRFRP3 disrupts the episodic release pattern of LH release in the ICS and suppresses GnRH-induced release of LH.

3.2.2.2 Specific objectives

Determine the effect of chronic administration of 250µg of eRFRP3, injected i.v. every 10 min for 6 h, on 1) the episodic pattern of LH release in mares during the follicular phase of the estrous cycle, and 2) GnRH-mediated release of LH. The dose of eRFRP3 was determined on the basis of BW by extrapolation from previous studies in smaller species as indicated in *3.2.1.5*.

3.2.2.3 Animals and study location

Thirteen Quarter Horse mares, ranging from 350-590 kg and 2-17 yr of age, were maintained on pasture (Coastal Bermuda grass) at Texas AgriLife Research-Beeville and supplemented with a mixed grain concentrate (12% crude protein; Falls City Milling, Falls City, TX) to maintain body condition score between 5 and 6 in a scale from 1 to 9. The day of ovulation was determined as described in *3.2.1.3*. On day 7 after ovulation, mares were treated intramuscularly (i.m.) with 2 mL prostaglandin F2α (PGF; 10 mg Lutalyse, Pfizer, New York, NY) to regress the corpus luteum (CL). The following day, the intercavernous sinus (ICS) of each mare was catheterized via the superficial facial vein using aseptic technique (Fig. 4). Mares were assigned randomly to one of two groups: 1) Control: 3 mL saline i.v. every 10 min for 8 h (n=6); or 2) eRFRP3 (n=7); saline every 10 min for 2 h (Period I), followed by 250 µg eRFRP3 in 3 mL saline every 10 min for 6 h (Period II). Six milliliter blood samples were collected at 5-min intervals for 6 h. At min 365 all mares were then administered a single i.v. injection of 1 mg GnRH and ICS blood samples were collected at 15 min intervals for an additional 2 h (Period III; Fig. 4).

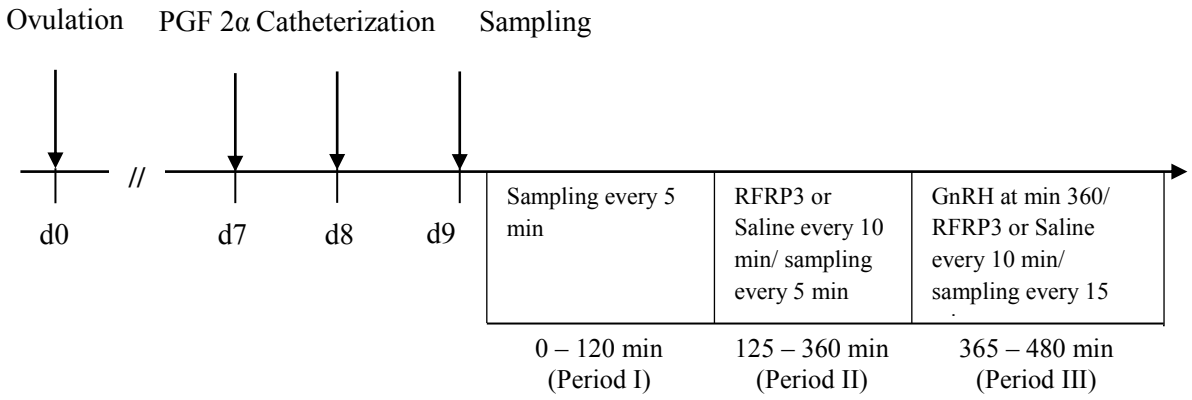

Fig. 4. Time line for experiment relative to day of ovulation and for experiment relative to sampling period.

On the day of sampling, mares were tied loosely, and provided water and hay during the sampling period. A volume of 6 mL of ICS blood was collected every 5 min. Mares were heparinized (40,000 IU sodium heparin, i.v.) at the beginning of the sampling period and also had the cannula flushed with 1.5 mL of heparinized saline (300 IU/mL) after each sample was collected. Samples were placed into tubes containing 50µL of a 5% EDTA-heparin solution (10,000 IU/mL) to prevent coagulation, placed immediately on ice and centrifuged for plasma collection every hour after harvesting. Plasma was stored at -20º C until analysis of LH. ICS cannulation was performed as described in *3.2.2.4*.

3.2.2.4 Intercavernous sinus (ICS) catheterization procedure

On the day following regression of the CL, the ICS was catheterized as describe previously by Irvine et al., Cooper et al. and Velez et al. [11, 13, 39] for assessing the pulsatile pattern of LH release in anterior pituitary venous effluent. The mare was placed

in a stock and sedated with detomidine HCl (0.5 to 1 mL of 10 mg/mL of solution (20-40 μg/kg BW)). The area that lies parallel and along the anterior border of the mandible, over the facial vein and extending in all directions (approximately 2 to 2.5 cm), was clipped, scrubbed, and disinfected for aseptic surgery. The facial vein was palpated through the skin and an area below the facial crest, which overlies and surrounds the vein (approximately 1.5 cm x 1.5 cm), was infiltrated subcutaneously with Lidocaine HCl (2%, Vedco, Inc, St Joseph, MO). A skin incision measuring approximately 1 cm was made over the vein, and the vein was exteriorized with a blunt dissection and held in this position by placing a sterile rod between the vein and the underlying tissue. A small incision was made in the vein using the point of the scalpel blade (number 10) and Tygon tubing measuring 38 cm (Norton Performance Plastics CO, Akron, OH; 0.1 cm ID x 0.2 cm OD), with a flexible J-straight guide wire inserted into, were inserted into the vein up to the point where resistance was felt. The resistance point usually occurs after 18 and 20 cm of tubing has been inserted. Once in place, a lateral radiograph was taken to visualize the position of the catheter and verify its location just behind and below the eye. Adjustments in catheter position were made as required. The guide wire was removed and a heparin (10,000 IU/mL) lock was placed in the tubing. The tubing was sutured to the edge of the incision with synthetic, non-absorbable suture above and below its exit. The skin incision was sutured partially closed and the wound is treated with an antiseptic skin dressing. The catheter was removed immediately after completion of intensive sampling. The skin area in front of the mandible where the tubing was

located was then treated daily with a topical antiseptic. Sutures were removed after 7 to 10 d.

3.2.2.5 Hormone analysis

Radioimmunoassay for LH was the same as for experiment 1. Mean intra- and inter-assay CV averaged 6.8% (±1.3) and 5.8%, respectively.

3.2.2.6 Statistical analysis

Because available pulse detection algorithms have not proven reliable in our hands for accurately detecting pulsatile release patterns of LH in horses, a different approach for detecting significant secretory episodes was employed. Luteinizing hormone data were transformed to a 3-point rolling average which reduced background noise. To be considered an episode, the coefficient of variation (CV) of the ascending and the descending sides of pultative episodes were required to be greater than the intra-assay CV. Once the episodes were identified, amplitude, frequency, and duration of each episode were determined. An expression for area under the curve for concentrations of LH after GnRH injection was delivered was as follows:

$$\int_a^b f(x)\,dx$$

The area under the curve created between y=f(x) axis and x-axis was given by the definite integral above. Values for *a* and *b* were determined by where the curve was located in the x-axis.

Data were analyzed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) to determine the effects of RFRP3 treatments on frequency, duration, and amplitude

of the episodes and the effect of GnRH challenge on the area under the LH curve. The statistical model for the repeated measures analyses included treatment as the main effect, period or time by treatment, and mare within treatment as the subject.

3.2.3 Experiment 3

Ability of a suprapharmacological dose of eRFRP3 to suppress secretion of LH in anovulatory mares treated continuously with GnRH.

3.2.3.1 Hypothesis

A single bolus treatment with eRFRP3 administered at a suprapharmacological dose will suppress secretion of LH stimulated by continuous administration of native GnRH.

3.2.3.2 Specific objectives

Determine whether 5 mg of eRFRP3, injected i.v. in a single bolus can suppress the pulsatile pattern of LH in winter anovulatory mares induced to secret LH by continuous administration of native GnRH.

3.2.3.3 Animals and study location

Six Quarter Horse mares ranging from 320-410 kg of body weight and 4-26 yr of age were maintained on pasture (Coastal Bermuda grass) at the Experiment Station and supplemented with a mixed grain concentrate (12% crude protein; Falls City Milling, Falls City, TX) to maintain body condition score between 5 and 6 on a scale of 1 to 9. Mares were confirmed as anovulatory by daily transrectal ultrasonography to monitor ovarian events, and analyses of serum progesterone concentrations for at least one month. For the mares to be considered anovulatory serum concentration of progesterone

was required to be lower than 1 ng/mL during this period with no ovarian follicles >30 mm. Mares were assigned in a replicated Latin square design (Table 2) to receive saline or eRFRP3 (5 mg i.v) in saline in random order on consecutive days, with a 24 h washout period between treatment/sampling. Treatments were injected at Time 0 and ICS blood samples were collected at 5-min intervals for 3 h following the post harvesting procedure described in Exp. 2. Samples were assayed for LH as in Exp. 1 and 2 to assess the episodic pattern and mean concentrations of LH.

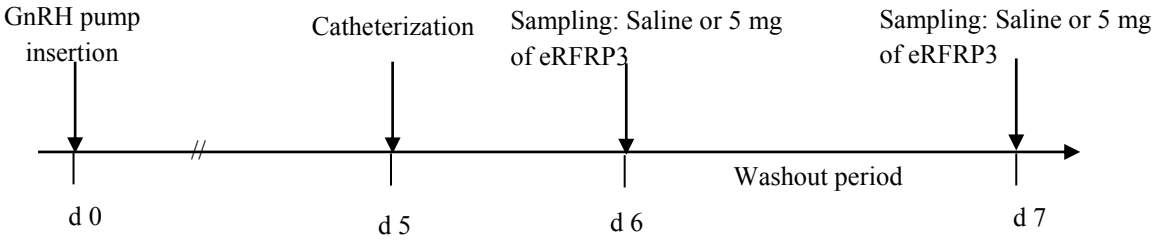

Fig. 5. Time line for experiment relative to GnRH pump insertion and to sampling period.

Table 2: Schedule for experiment relative to sampling period.

Replicate	Mare	Day 1	Day2
1	1 and 2	C-mare1/ RFRP3- mare2	RFRP3-mare1/ C-mare2
2	3 and 4	RFRP3-mare3/ C-mare4	C-mare3/ RFRP3-mare4
3	5 and 6	C-mare5/ RFRP3-mare6	RFRP3-mare5/ C-mare6

Treatments: C= saline; RFRP3= 5 mg of RFRP3.

3.2.3.4 Hormone analysis

Hormone analysis for LH was the same as for experiment 1 and 2. Mean intra- and inter-assay CV were 10.8% (±2.5) and 10.3%, respectively. Concentrations of progesterone in serum were measured using a commercial single antibody kit (Diagnostic Products Corporation, Los Angeles, CA) for samples collected daily. All samples were analyzed for progesterone in a single assay with minimum detectable concentration of 0.1 ng/ml.

3.2.3.5 Statistical Analysis

Because of the inability to detect secretory episodes of LH using the methodology described in Exp.2, only mean concentrations of LH were statistically-analyzed and analyses were performed as described in Exp.1.

3.2.4 Experiment 4

Determine the effects of a suprapharmacological dose of ovine RFRP3 (oRFRP3) on the secretion of LH in mares in the follicular phase.

3.2.4.1 Hypothesis

A single bolus dose of oRFRP3 administered at a suprapharmacological level will suppress secretion of LH.

3.2.4.2 Specific objectives

Determine the ability of 5 mg of oRFRP3, injected i.v. in a single bolus in the suppression of the mean concentration and pulsatile pattern of LH in mares during follicular phase of the estrous cycle.

3.2.4.3 Animals and study location

Six Quarter Horse mares ranging from 360-500kg of body weight and 3-15 yr of age were maintained on pasture (Coastal Bermuda grass) at the Experiment Station and supplemented with a mixed grain concentrate (12% crude protein; Falls City Milling, Falls City, TX) to maintain body condition score between 5 and 6 in a scale from 1 to 9. The day of ovulation and CL regression (Fig. 6) were the same as described on *3.2.1.3* and *3.2.2.3*. Mares were assigned in completely randomized design to receive saline (n=3) or oRFRP3(n=3) (10 µg/kg of BW delivered i.v, with the total dose ranging from 3mg to 5mg depending on the size of the mare). Samples were collected from the ICS cannula at 5-min intervals for 3h. At 180 min, saline or oRFRP3 was injected and samples were collected for 3 more h following the same procedure for blood processing as described in Exp. 2 and 3. Samples were assayed for LH as in Exp. 1, 2, and 3 to determine mean concentrations of LH.

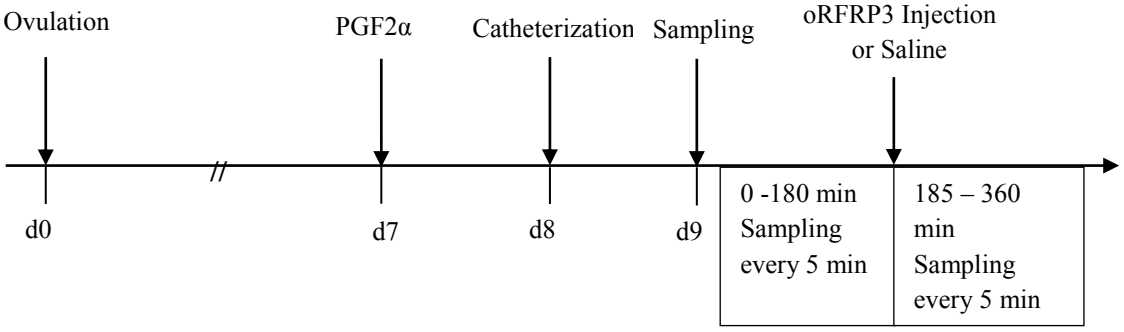

Fig. 6. Time Line for experiment relative to day of ovulation and for experiment relative to sampling period.

3.2.4.4 oRFRP3 sequence synthesis

The ovine sequence (NH2-Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-Amide), provided by Dr. Terry Nett, Colorado State University, was synthesized and chemically validated (High-performance liquid chromatography and Mass spectral analysis (95% of purity and 969 of molecular weight)) by United Biochemical Research, Inc. (Seattle, WA,US).

3.2.4.5 Hormone analysis

The RIA for LH was the same as for experiment 1, 2, and 3. Mean intra- and inter-assay CV were 13.2 (±3.8) and 12.2%, respectively.

3.2.4.6 Statistical Analysis

Because of an inability to detect episodic release of LH in ICS samples from mares used in this experiment, only mean concentrations of LH were compared using the same procedure described in Exp.2 excluding the other variables not measured in this case.

3.3 Results

3.3.1 Experiment 1

Figure 7 represents least-squares mean concentrations of LH for mares in each experimental group during the 7-day GnRH infusion (20 μg/h) period leading up to experimental treatments with RFRP3 or saline (control) on Day 7. Mean concentrations determined from single daily jugular samples did not differ among groups (P=0.99). Figure 8 shows mean concentration of LH during the 4-h intensive sampling period (samples collected every 15 min), pooled by hour, before and after onset of RFRP3 and saline (control) treatments. Since mean baseline concentrations of LH differed among

the 3 groups at the onset of sampling period, data are normalized as the percentage of time zero values. Mean concentrations of LH were not affected by treatment (P=0.99).

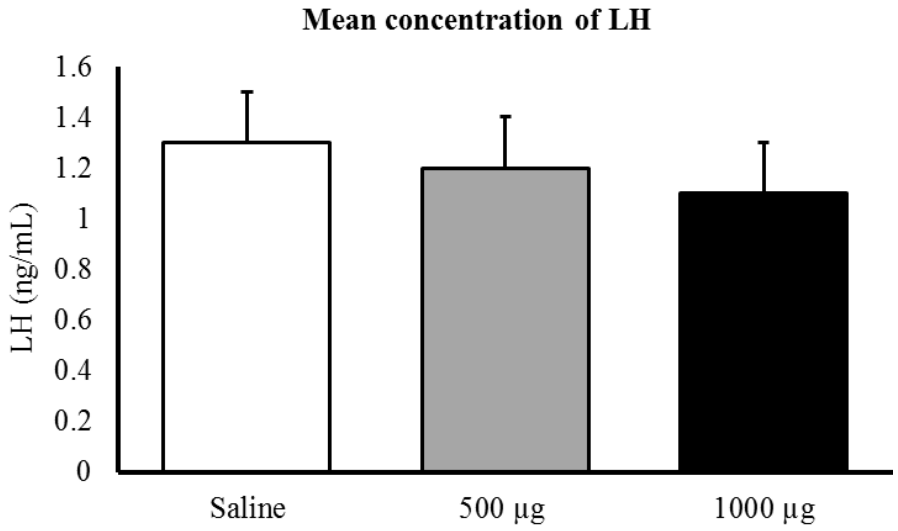

Fig. 7. LSmean concentrations of LH (ng/mL ± SEM) in each experimental group during the 7-d GnRH infusion period before experimental treatments.

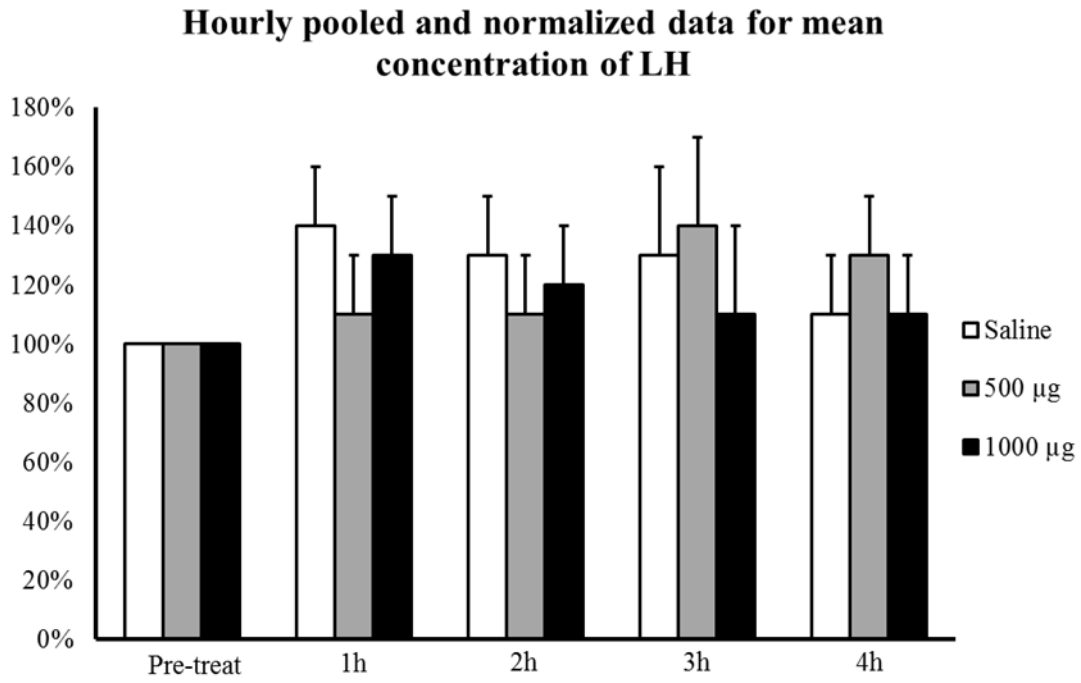

Fig. 8. Normalized mean concentration of LH (% of time zero) in saline (control) and eRFRP3-treated (500 and 1000 µg) mares pooled by hour from samples collected every 15 min.

3.3.2 Experiment 2

Episodic secretion patterns of LH in 2 representative control and 2 representative eRFRP3-treated mares are shown in Fig. 9. Areas under the GnRH-induced LH curves for each treatment are shown in Fig. 10. Treatment with eRFRP3 (250 µg/h) every 10 min for 4 h before GnRH challenge and continuing for 2 h after GnRH challenge had no effect on any variables measured, including mean concentration (P=0.35), frequency (P=0.23), amplitude (P=0.60), and duration (P=0.25) of LH secretory episodes, nor on areas under the GnRH-induced release of LH (P=0.31; Table 3).

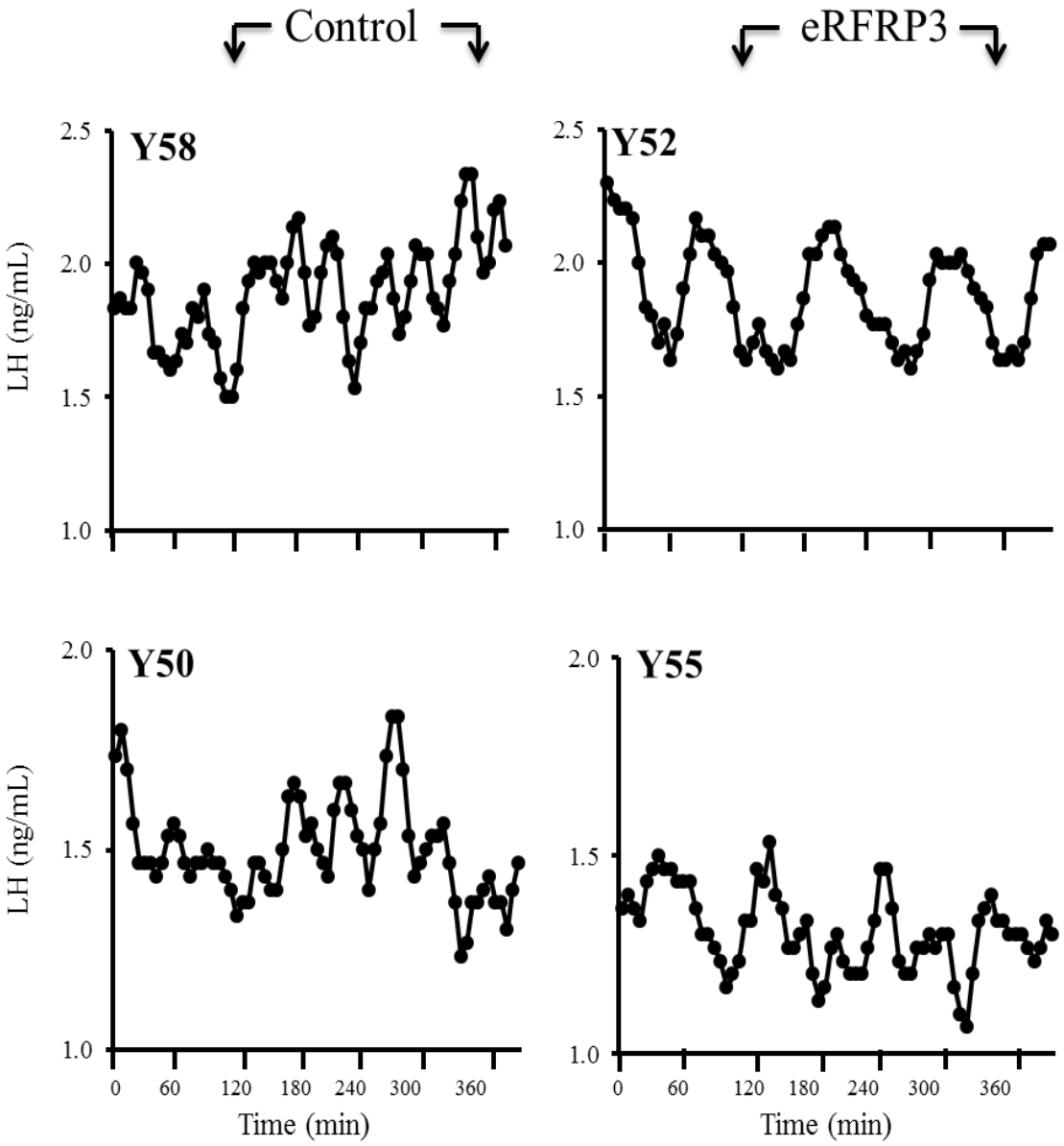

Fig. 9. Pattern of secretion of LH for 2 representative control (left panels) and 2 representative eRFRP3-treated mares (right panels). Mares were injected i.v. with either saline or 250 μg eRFRP3 every 10 min for 4 h beginning 60 min after the start of sampling.

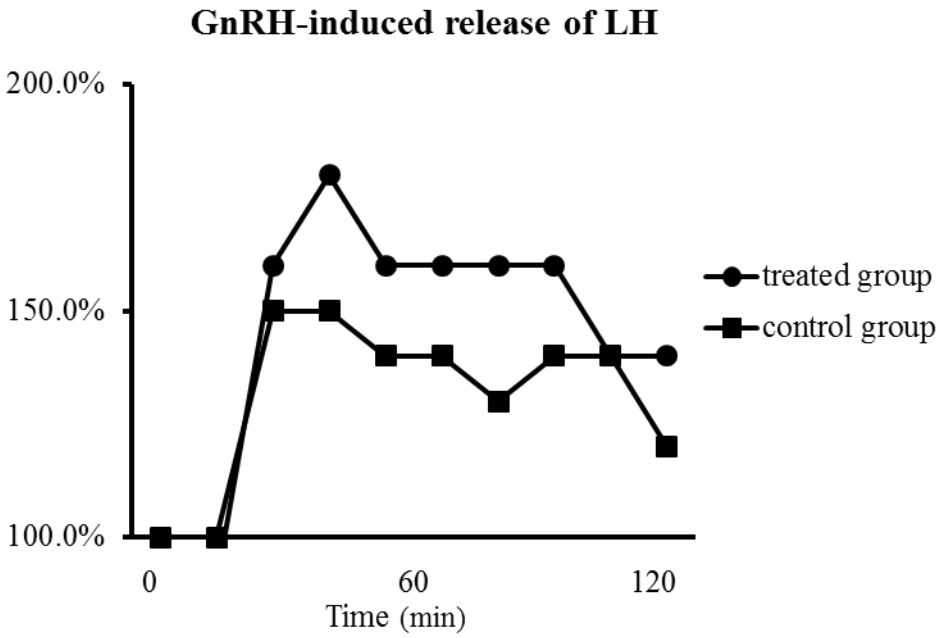

Fig. 10. GnRH-induced release of LH in mares treated with GnRH (1 mg i.v.) at time 365 min (represented as time 0) of intensive sampling. Samples were collected every 15 min post GnRH-infusion for 2 h.

Table 3: LSmean (± SEM) concentrations of LH, frequencies, amplitudes, and durations of LH secretory episodes before (Period 1) and during (Period 2) treatment with saline or eRFRP3, and GnRH-induced release of LH. Saline or RFRP3 (250µg/injection) was injected at 10-min intervals for 4 h before GnRH treatment and continued for an additional 2 h after a bolus i.v. injection of 1mg GnRH. No effects of eRFRP3 were detected.

	Treatments			
	Control		eRFRP3	
Period*	1	2	1	2
LH (ng/mL)	1.8 ± 1.2	1.9 ± 1.4	1.3 ± 0.5	1.3 ± 0.4
Amplitude (ng/mL)	0.3 ± 0.02	0.3 ± 0.02	0.2 ± 0.03	0.2 ± 0.02
Frequency	2.8 ± 0.5	5.3 ± 0.5	2.1 ± 0.5	4.0 ± 0.5
Duration (min)	35. 2 ± 2.7	33.6 ± 2.1	40.4 ± 2.9	46.1 ± 2.2
GnRH-induced release of LH (Area under the curve)**	175.9±11.4		192.6±10.6	

* Period 1 – before treatment; Period 2- after treatment
** Arbitrary units

3.3.3 Experiment 3

This experiment examined the ability of a very large dose of eRFRP3, administered as a single bolus i.v. injection, to suppress the episodic release of LH in the ICS of winter anovulatory mares infused continuously with GnRH (100 µg/h) for 7 d. The patterns of secretion of LH from 2 representative saline-treated control and 2 eRFRP3-treated mares, respectively, are shown on Fig. 11. Overall least-squares mean concentrations of LH for both treatments are shown in Fig. 12. Treatment with eRFRP3 had no effect on secretion of LH (P=0.66) in this experiment.

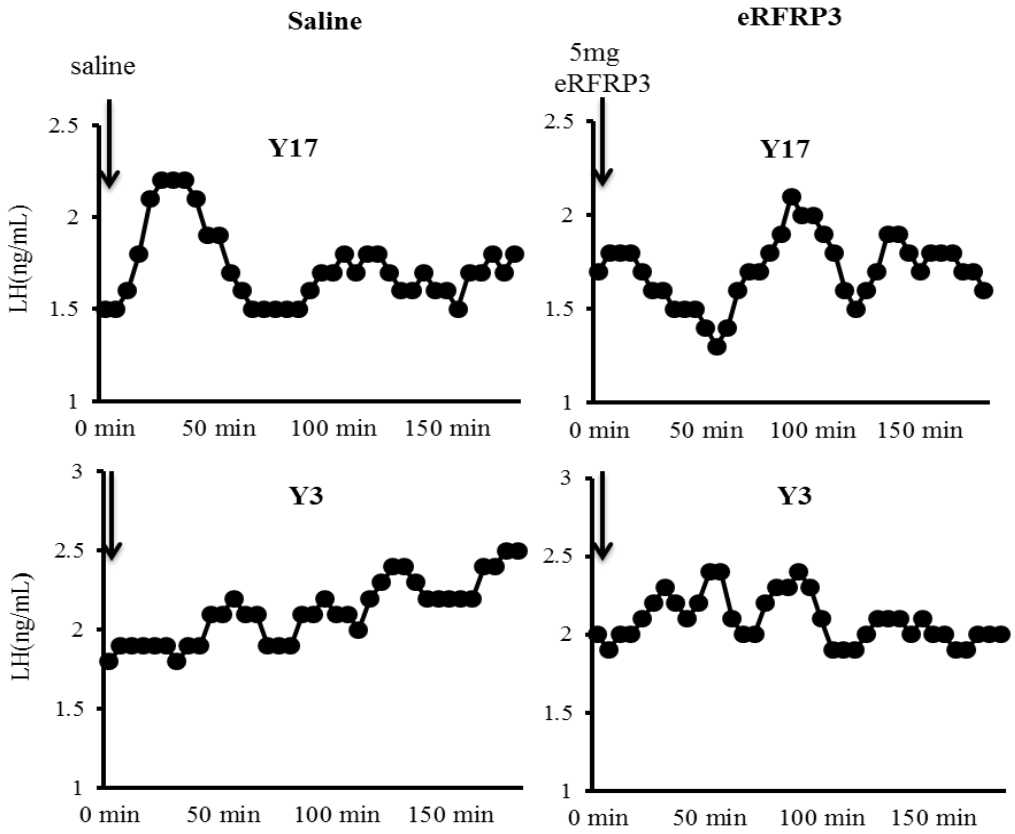

Fig. 11. LH secretory pattern plotted using three-point rolling average of 5 min samples collected over 3-h for representative mares during the treatment and control period.

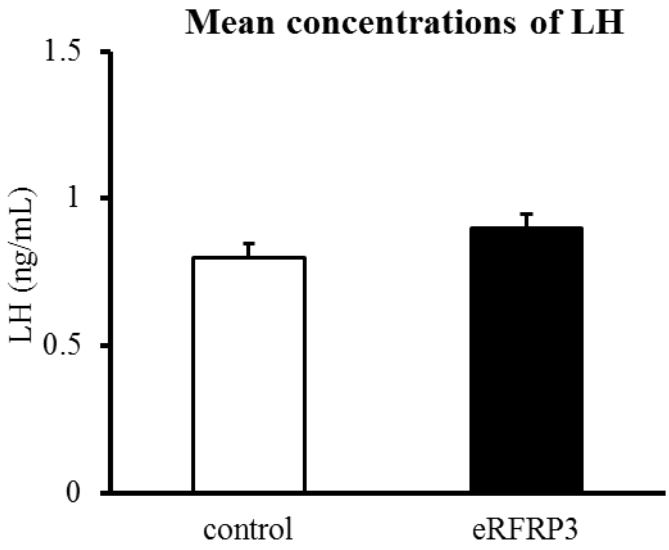

Fig. 12. Mean concentrations of LH in mares administered saline control or 5 mg eRFRP3 i.v.

3.3.4 Experiment 4

Because no effects of eRFRP3 were noted in Exp. 1-3, a final study was done utilizing an ovine RFRP3 as a positive control. Previously, the ovine homolog of the avian RFRP3 has been shown to suppress secretion of LH in sheep. However, treatment of follicular phase mares with oRFRP3 (10µg/kg of BW) as a single i.v. bolus injection failed to result in measurable decrease in mean concentrations of LH (P=0.58) in ICS plasma (Fig. 13).

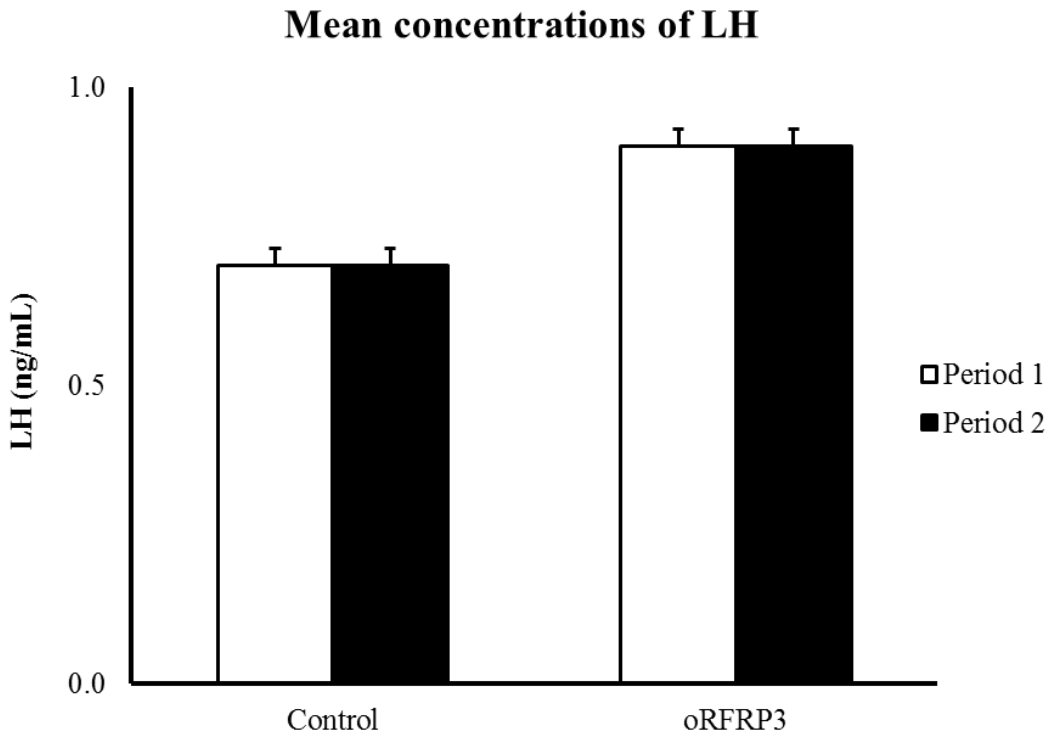

Fig. 13. Mean concentrations of LH for mares in saline control (n=3) and oRFRP3 (10µg/kg of BW; n=3) groups for 3 h before (Period 1) and 3 h after (Period 2) treatment. ICS concentrations of LH were determined in blood samples collected at 5-min intervals.

3.4 Discussion

The discovery and characterization of avian GnIH [14, 17, 24, 29, 30, 130, 131] and mammalian RFRP3 [17, 18, 19, 20, 135] has resulted in significant evidence to indicate that this peptide is able to suppress the secretion of LH and is involved in the control of reproductive seasonality in birds [131], and perhaps some mammals [18]. However, none of these studies were conducted in the mare, a species which is profoundly seasonal and in which seasonality has significant economic ramifications.

In the current studies, we set out to examine if RFRP3 could contribute to the mechanisms that control seasonality in the mare. Results indicate that neither eRFRP3 at any dose or manner tested, nor oRFRP3 as a large single dose, is able to suppress episodic release, mean concentration, or GnRH-induced release of LH in mares. Thus, our studies were unable to support the hypothesis that RFRP3 acts as an inhibitory signal that can suppress the secretion of LH through effects at the hypothalamic or pituitary gonadotrope level in the mare.

In the first of four experiments, we used a model that compared the ability of 500 and 1,000μg of eRFRP3 to suppress peripheral concentrations of LH during the luteal phase of mares treated continuously with GnRH. The continuous infusion of GnRH was utilized because previous studies in our lab [11] have shown that such treatments markedly increase the LH baseline and create a consistent pattern of high-frequency pulses. Thus, this eliminates the need to synchronize mares to a particular phase of the cycle and avoids the infrequent, long-duration episodes of LH release that are observed during the normal luteal phase [167, 168, 169, 170]. The latter would make it quite difficult to contrast RFRP3 effects in mares due to asynchronous occurrences of large episodes of LH. As shown in Figure 9, the eRFRP3 treatments did not suppress mean concentration of LH. Although, earlier studies have shown that intravenously-administered RFRP3/GnIH is effective in suppressing circulating concentrations of LH in rats [35], in Syrian hamsters [20], and in male song sparrows [131]. Furthermore, RFRP3 suppressed secretion of LH in female rat pituitary cells cultured in the presence of GnRH [35].

For the second experiment, we used mares in the follicular phase of estrous cycle and treated them with eRFRP3 every 10 min for 6 h (total dose of 9 mg of RFRP3). Treatment did not affect duration, frequency, or amplitude of LH release episodes or mean concentration measured in intensively-collected ICS sampled. The ICS cannulation technique was chosen for this experiment to facilitate detection of LH secretory episodes that otherwise cannot be detected in peripheral plasma [167]. Mares where challenged with 1 mg of GnRH after 360 min of intensive sampling in order to induce maximal release of LH from the anterior pituitary. The resulting increment in LH was similar to that observed previously when mares on the second or third day of the estrous cycle were treated intravenously with GnRH at a dose of 2μg/kg BW. Treatment with RFRP3 was ineffective in modifying GnRH-induced release of LH. As in a previous study, the concentration of LH in plasma never returned to baseline during the sampling period [69]. Earlier studies in ruminants have included male calves injected with RFRP3 (90μg) every 10 min for 1 h [33] and ovariectomized ewes given a loading dose of 50 μg followed by a continuous infusion of 200 μg for over 1 h [18]. Both studies indicated that RFRP3 suppressed pulsatile release of LH. However, when ovariectomized prepubertal gilts were given a loading dose of 1mg of RFRP3 and then treated with 40μg of RFRP3 [intracerebroventricular, i.c.v] every 5 min for 2 h, neither mean concentrations of LH nor the number or amplitude of LH pulses were altered [171]. Moreover, area under the curve was also calculated for the period before and after RFRP3 treatment and no statistical difference was observed for total LH release between periods. Experiments have also been conducted in rodents. When ovariectomized,

estradiol-replaced rats were treated i.c.v with RFRP3, no effects on the pattern of LH release were observed [32]. However, in another study with ovariectomized female rats, i.v. treatment with 1 or 10μg of RFRP3, in combination with either saline or GnRH, reduced peak LH at minute 5 after GnRH [144]. Furthermore, secretion of LH was inhibited in pituitary cell cultures of male quail [29], rat [35], chicken [146], and sheep [18] treated with RFRP3. Thus, effects of RFRP3 in mammalian species have been somewhat mixed. With respect to ruminant species, at least two other studies in sheep have been conducted without confirmation of significant RFRP3 effects [Drs. Terry Nett, Colorado State University and Dr. Alain Caraty, INRA, Nouzilly, France, personal communications].

In the third experiment reported herein, mares were confirmed anovulatory and were administered an Alzet osmotic pump delivering GnRH s.c at a rate of 100μg/h of for 7 d. Previous studies have shown repeatedly that continuous GnRH treatment stimulates the secretion of LH from the anterior pituitary during the non-breeding season [11, 172, 173, 174]. Treatment with a bolus injection of 5mg RFRP3 failed to affect mean concentrations of LH compared to saline controls in samples collected from the ICS. Unfortunately, the degree of stimulation of the LH baseline in this study was not adequate to allow reliable detection of the episodic release of LH in these mares. In rats treated with estradiol to induce the release of GnRH/LH, both 2.5 and 25 ng of RFRP3 (i.c.v) suppressed activation of 70% of anteroventral periventricular GnRH cells [32]. However, the investigators were not able to demonstrate a significant decrease in plasma concentration of LH [32]. In cultured ovine pituitary cells treated with GnRH, oRFRP3,

or GnRH+oRFRP3, a dose dependent inhibition of LH was observed only when oRFRP3 was applied to cells in the presence of GnRH [18]. Similarly, a study conducted in photorefractory song sparrows showed that secretion of LH was attenuated more in the group treated with GnRH than in the group treated with GnRH plus 1,000μg of quail GnIH [131].

Finally, in the last experiment reported, we tested the hypothesis that mares in the follicular phase treated with an ovine sequence of RFRP3 (NH2-Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-Amide) would exhibit suppression of LH release similar to that reported previously in ovariectomized ewes [18]. Thus, this experiment served as a positive control using a peptide sequence shown previously to have significant biological effects across multiple mammalian species [18, 33, 34, 35, 137]. It appears that the last 3 amino acids of all homologs are identical across species and confer biological activity to the molecule. For example, when white-crowed sparrows were castrated during their non-breeding season, photostimulated, and injected with 500μg of quail GnIH, mean plasma concentrations of LH were significantly reduced within 3 min after injection [131], demonstrating the heterospecificity of this peptide. The dose of RFRP3 chosen for our experiment was 10μg/kg of BW, which is similar to that reported previously in studies where RFRP3 suppressed LH [18, 33]. Similar to Exp. 3, we were not able to measure the episodic release of LH in the pituitary venous effluent and only mean concentrations of LH were compared. Mares used in this experiment had relatively low baseline concentrations of LH that were not characterized by robust pulses. No effects of oRFRP3 were observed on mean concentration of LH in samples collected at 5-min

intervals from the ICS. The ovine sequence utilized in this experiment has been shown to be efficient in suppressing the pulsatile secretion pattern of LH as well as LH release from cultured ovine pituitary cells and in an *in vivo* experiment [18]. However, the preparation itself, obtained from Dr. Terry Nett, Colorado State University, was shown to have no effect on pulsatile release of LH in ewes.

CHAPTER IV

CONCLUSION

Results obtained in the four experiments described herein indicate that the equine hypothalamic-gonadotropic axis is not responsive to RFRP3 as shown previously in some other experiments with birds and mammals. Thus, we were not able to provide evidence of a contribution of RFRP3 to reproductive seasonality in the mare. Infusion of both eRFRP3 and oRFRP3 did not disrupt either the normal pattern of LH release, GnRH-induced release of LH, or mean concentration of LH as demonstrated to occur in earlier studies with other seasonal species. Therefore, results do not support a role for RFRP3 in regulating the reproductive neuroendocrine axis of the mare.

REFERENCES

[1] Ginther OJ. Reproductive biology of the mare: basic and applied aspects. Cross Plains, WI: Equiservices; 1992:105-172.

[2] Fitzgerald BP, Schmidt MJ. Absence of an association between melatonin and reproductive activity in mares during the nonbreeding season. Biol Reprod Mono. 1995; (1):425-434.

[3] Alexander SL, Irvine CHG. GnRH secretion in the mare. Animal Reproduction Science 1996; (42):173-180.

[4] Sharp DC, Kooistra L, Ginther OJ. Effects of artificial light on the oestrous cycle of the mare. J Reprod Fertil Suppl. 1975; (23):241-246.

[5] Strauss SS, Chen CL, Kalra SP, Sharp DC. Depletion of hypothalamic gonadotropin-releasing hormone (GnRH) in ovariectomized pony mares following melatonin implants. Fed. Proc. 1978; Suppl. (37):225.

[6] Hotchkiss J, Knobil E. The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd Edition, Vol. 2. New York: Raven Press; 1994: 711-749.

[7] Goodman RL. Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd Edition, Vol. 2. New York: Raven Press; 1994: 659-709.

[8] Freeman MC. The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd Edition, Vol. 2. New York: Raven Press; 1994: 613-658.

[9] Becker SE, Johnson AL. Effects of gonadotropin-releasing hormone infused in a pulsatile or continuous fashion on serum gonadotropin concentrations and ovulation in the mare. J Anim Sci. 1992; (70):1208-1215.

[10] McCue PM, Troedsson MHT, Liu IKM, Stabenfelt GH. Follicular and endocrine responses of anoestrous mares to administration of native GnRH or a GnRH agonist. J Repro Fert Suppl. 1991; (44):227-233.

[11] Velez IC, Pack JD, Porter MB, Sharp DC, Amstalden M, Williams GL. Secretion of luteinizing hormone into pituitary venous effluent of the follicular and luteal phase mare:

novel acceleration of episodic release during constant infusion of gonadotropin-releasing hormone. Domest Anim Endocrinol. 2012; Article in press.

[12] Hyland JH, Jeffcott LB. Control of transitional anestrus in mares. Theriogenology 1988; (296):1383-1391.

[13] Cooper DA. Reproductive neuroendocrine function in the mare as reflected in the intercavernous sinus during ovulatory, anovulatory, and transitional seasons. M.S. Thesis, Texas A&M University, 2006

[14] Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S, Sharp PJ; A novel avian hypothalamic peptide inhibiting gonadotropin release.; Biochem Biophys Res Commun. 2000; (275):661-667.

[15] Matteri, RL., Moberg, GP. The effect of opioid peptides on ovine pituitary gonadotropin secretion in vitro. Peptides 1985; (6):957-963.

[16] Blank MS, Fabbri A, Catt KJ, Dufau ML. Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells. Endocrinology 1986; (118):2097-2101.

[17] Bentley GE, Kriegsfeld LJ, Osugi T, Ukena K, O'Brien S, Perfito N, Moore IT, Tsutsui K, Wingfield JC. Interactions of gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) in birds and mammals. J Exp Zoolog A Comp Exp Biol. 2006; (305):807-814.

[18] Clarke IJ, Sari IP, Qi Y. Potent action of RFRP-3 on pituitary gonadotropes indicative of an hypophysiotropic role in the negative regulation of gonadotropin secretion. Endocrinology 2008; (149):5811-5821.

[19] Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nature Cell Biology 2000; (2):703-708.

[20] Kriegsfeld LJ, Mei DF, Bentley GE. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. Proc Natl Acad Sci USA 2006; (103):2410-2415.

[21] Sawada K, Ukena K, Kikuyama S, Tsutsui K. Identification of a cDNA encoding a novel amphibian growth hormone-releasing peptide and localization of its transcript. J Endocrinol. 2002; (174):395-402.

[22] Amano M, Moriyama S, Iigo M, Kitamura S, Amiya N, Yamamori K, Ukena K**,** Tsutsui K. Novel fish hypothalamic neuropeptides stimulate the release of

gonadotrophins and growth hormone from the pituitary of sockeye salmon. Journal of Endocrinology 2006; (188):417-423.

[23] Tsutsui K, Ukena K. Hypothalamic LPXRF-amide peptides in vertebrates: identification, localization and hypophysiotropic activity. Peptides 2006; (27):1121-1129.

[24] Ikemoto T, Park MK. Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: identification, molecular characterization, and evolutionary considerations. J Reprod Dev. 2005; (51):359-377.

[25] Ukena K, Tsutsui K. A new member of the hypothalamic RF-amide peptide family, LPXRF-amide peptides: structure, localization, and function. Mass Spectrom Rev. 2005; (24):469-486.

[26] Sawada K, Ukena K, Satake H, Iwakoshi E, Minakata H, Tsutsui K. Novel fish hypothalamic neuropeptide: Cloning of a cDNA encoding the precursor polypeptide and identification and localization of the mature peptide. Eur J Biochem. 2002; (269):6000-6008.

[27] Ukena K, Tsutsui K. A new member of the hypotalamic RFamide peptide family, LPXRF-amide peptides: structure, localization, and function. Wiley InterScience 2005; DOI 10.1002/mas.20031.

[28] Yoshida H, Habata Y, Hosoya M, Kawamata Y, Kitada C, Hinuma S. Molecular properties of endogenous RFamide-related peptide-3 and its interaction with receptors. Biochimica Biophysica 2003; (1593):151-157.

[29] Ubuka T, Ukena K, Sharp PJ, Bentley GE, Tsutsui K. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. Endocrinology March 2006; (147):1187-1194.

[30] Ukena K, Ubuka T, Tsutsui K. Distribution of a novel avian gonadotropin-inhibitory hormone in the quail brain. Cell Tissue Res. 2003; (312):73-79.

[31] Ubuka T, Kim S, Huang YC, Reid J, Jiang J, Osugi T, Chowdhury VS, Tsutsui K, Bentley GE. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. Endocrinology 2008; (149):268-278.

[32] Anderson GM, Relf HL, Rizwan MZ, Evans JJ. Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. Endocrinology 2009; (150):1834-1840.

[33] Kadokawa H, Shibata M, Tanaka Y, Kojima T, Matsumoto K, Oshimab K, Yamamoto N. Bovine C-terminal octapeptide of RFamide-related peptide-3 suppresses luteinizing hormone (LH) secretion from the pituitary as well as pulsatile LH secretion in bovines. Domestic Animal Endocrinology 2009; (36):219-224.

[34] Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav. 2007; (51):171-180.

[35] Murakami M, Matsuzaki T, Iwasa T. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. J Endocrinol. 2008; (199):105-112.

[36] Sharp DC, Seamans KW. Effects of time of day on photostimulation of the breeding season in mares. J Animal Science 1980; (Suppl. 1): 328.

[37] Williams GL, Amstalden M, Blodgett GP, Ward JE, Unnerstal DA, Quirk KS. Continuous administration of low-dose GnRH in mares. I. Control of persistent anovulation during the ovulatory season. Theriogenology 2007; (68):67-75.

[38] Collins, SM, Zieba, DA, Williams, GL. Continuous administration of low-dose GnRH in mares II. Pituitary and ovarian responses to uninterrupted treatment beginning near the autumnal equinox and continuing throughout the anovulatory season. Theriogenology 2007; (68):673-681.

[39] Irvine CHG, Alexander SL. A novel technique for measuring hypothalamic and pituitary hormone secretion rates from collection of pituitary venous effluent in the normal horse. J. Endocrinol. 1987; (113):183.

[40] Kooistra LH, Ginther OJ. Effect of photoperiod on reproductive activity and hair in mares. Am. J. Vet. Res. 1975; (36):1413-1419.

[41] Palmer E, Driancourt MA, Ortavant R. Photoperiodic stimulation of the mare during winter anoestrus. J. Reprod. Fertil. 1982; (32):275-282.

[42] Scraba ST, Ginther OJ. Effects of lighting programs on onset of the ovulatory season in mares. Theriogenology 1985; (24):667-679.

[43] Burkhardt J. Transition from anestrus in the mare and the effects of artificial lighting. J. Agric. Sci. 1947; (37):64-68.

[44] Palmer E, Guillaume D. Photoperiodism in the equine species–what is a long night? Anim. Reprod. Sci. 1992; (28):21-30.

[45] Cox JE, Skidmore JL. The effect of alternating months of ‘‘long days’’ and ‘‘short days’’ on plasma luteinizing hormone and testosterone concentrations in Welsh Mountain Pony stallions. Anim. Reprod. Sci. 1991; (25):51-55.

[46] Palmer E, Driancourt MA. Photoperiodic stimulation of the winter aneostrous mare: what is a long day? Photoperiodism and Reproduction in Vertebrates 1981; (6):65-82.

[47] Malinowski K, Johnson AL, Scanes CG. Effects of interrupted photoperiods on the induction of ovulation in anestrous mares. J. Anim. Sci.1985; (61):951.

[48] Sharp DC, Ginther OJ. Stimulation of follicular activity and estrous behavior in anestrous mares with light and temperature. Anim. Sci. 1975; (41):1368-1372.

[49] Nagy P, Guillaume D, Daels P. Seasonality in mares. Animal Reproduction Science 2000; (60-61): 245-262.

[50] Henneke DR, Potter GD, Kreider JL. Body condition during pregnancy and lactation and reproductive efficiency of mares. Theriogenology 1984; (21):897-909.

[51] van Niekerk CH, van Heerden JS. Nutrition and ovarian activity of mares early in the breeding season. J. South Afr. Vet. Med. Assoc.1972; (43):351-360.

[52] Ginther OJ. Occurrence of anestrous, estrus, diestrus, and ovulation over a twelve-month period in mares. Am. J. Vet. Res. 1974; (35):1173-1179.

[53] McDaniel JB, Kreider JL, Thrasher DM. The influence of artificial light and a nutritional supplement on the onset of the breeding season in mares. J. Anim. Sci. 1979; (49):141.

[54] Kubiak JR, Crawford BH, Squires EL, Wrigley RH, Ward GM. The influence of energy intake and percentage body fat on the reproductive performance of nonpregnant mare. Theriogenology 1987; (28):587-598.

[55] Carnevale EM, Ginther OJ. Age and pasture effects on vernal transition in mares. Theriogenology 1997; (47):1009-1018.

[56] Malpaux B, Daveau A, Maurice-Mandon F, Duarte G, Chemineau P. Evidence that melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe: presence of binding sites and stimulation of luteinizing hormone secretion by in situ microimplant delivery. Endocrinology 1998; (139):1508-1516.

[57] Guillaume D, Palmer E. Effect of oral melatonin on the date of the first ovulation after ovarian inactivity in mares under artificial photoperiod. J. Reprod. Fertil. 1991; (Suppl. 44): 249-257.

[58] Guillaume D, Arnaud G, Camillo F, Duchamp G, Palmer E. Effects of melatonin implants on reproductive status of mares. Biol. Reprod. Monogr. 1995; (1):435-442.

[59] Wesson JA, Quay WB, Ginther OJ. Seasonal relationship between pineal hydroxyindole-*O*-methyl-transferase (HIOMT) activity and reproductive status in the pony. Gen. Comp. Endocrinol 1979; (38):46-52.

[60] Grubaugh WR, Sharp DC, Berglund LA, McDowell KJ, Kilmer DM, Peck LS, Seamans KW. Effects of pinealectomy in pony mares. J. Reprod. Fertil. 1982; (Suppl. 32):293-295.

[61] Sharp DC, Grubaugh WR, Berglund LA, Seamans KW. Isoproperenol-stimulation of melatonin release in mares. J. Anim. Sci. 1980; (51):534.

[62] Malpaux B, Thie´ry J-C, Chemineau P. Melatonin and the seasonal control of reproduction. Reprod. Nutr. Dev. 1999; (39):355-366.

[63] Garcia MC, Freedman U, Ginther OJ. Interaction of seasonal and ovarian factors in the regulation of LH and FSH secretion in the mare. J Reprod Fertil 1979; (Suppl.27): 103-111.

[64] Garcia MC, Ginther OJ. Regulation of plasma LH by estradiol and progesterone in ovariectomized mares. Biol Reprod 1978; (19):447-453

[65] Garcia MC, Ginther OJ. Effects of ovariectomy and season on plasma luteinizing hormone in mares. Endocrinology 1976; (98):958-962.

[66] Freedman Li, Garcia MC, Ginther OJ. Influence of photoperiod and ovaries on seasonal reproductive activity in mares. Biol Reprod 1979; (20):567-574.

[67] Turner DD, Garcia MC, Ginther OJ. Follicular and gonadotropic changes throughout the year in pony mares. Am J Vet Res 1979; (40): 1694-1700.

[68] Garcia MC, Ginther OJ. Effects of ovariectomy and season on plasma luteinizing hormone in mares. Endocrinology 1976; (98):958-962.

[69] Silvia PJ, Squires EL and Nett TM. Pituitary responsiveness of mares challenged with GnRH at various stages of the transition into the breeding season. J. Anita. Sci. 1987; (64):790-796.

[70] Hart PJ, Squires EL, Imel KJ, Nett TM. Seasonal variation in hypothalamic content of gonadotropin-releasing hormone (GnRH), pituitary receptors for GnRH, and pituitary content of luteinizing hormone and follicle-stimulating hormone in the mare. Biol Reprod 1984; (30):1055-1062.

[71] Strauss SS, Chen CL, Kalra SP, Sharp DC. Localization of gonadotropin-releasing hormone (GnRH) in the hypothalamus of ovariectomized pony mares by season. J Reprod Fertil 1979; (27):123-129.

[72] Alexander SL, Irvine CHG. Effect of graded doses of gonadotropin-releasing hormone on serum LH concentration in mares in various reproductive states: comparison with endogenously generated LH pulses. Journal of Endocrinology 1986; (110):19-26.

[73] Sharp DC, and Grubaugh WR. Use of push-pull perfusion techniques in studies of gonadotrophin-releasing hormone secretion in mares. J. Reprod. Fert.1987; (Suppl. 35):289-296.

[74] Thiery JC, Malpaux B. Seasonal regulation of reproductive activity in sheep. Ann N.Y. Acad Sci 2003; (1007):169-175.

[75] Moenter SM, Woodfill CJI, Karsch FJ. Role of the thyroid gland in seasonal reproduction: thyroidectomy blocks seasonal suppression of reproductive neuroendocrine activity in ewes. Endocrinology 1991; (128):1337-1344.

[76] Thrun LA, Dahl GE, Evans NP, Karsch FJ. Effect of thyroidectomy on maintenance of seasonal reproductive suppression in the ewe. Biol Reprod 1997; (56):1035-1040.

[77] Viguie C, Battaglia DF, Krasa HB, Thrun LA, Karsch FJ. Thyroid hormones act primarily within the brain to promote the seasonal inhibition of luteinizing hormone secretion in the ewe. Endocrinology 1999; (140):1111-1117.

[78] Johnson AL. Serum concentrations of prolactin, thyroxine and triiodothyronine relative to season and the estrous cycle in the mare. J Anim Sci 1986; (62):1012-1020.

[79] Lincoln GA, Hazlerigg DG. Mammalian circannual pacemakers. Soc Reprod Fertil Suppl. 2010; (67):171-86.

[80] Wickings EJ, Nieschlag E. Seasonality in endocrine and exocrine testicular function of the adult rhesus monkey (Macaca mulata) maintained in a controlled laboratory environment. Int J Androl 1980; (3):87-104.

[81] Wehr TA. Photoperiodism in humans and other primates: evidence and implications. J Biol Rhythms 2001; (16):348-364.

[82] Pengelley ET, Asmundson SJ. Circanual clocks: circannual rhythmicity in hibernating animals. San Francisco, CA: Academic Press; 1974.

[83] Mrosovsky N. Strategies in cold: natural torbidity and thermogenesis: circannual cycles in hybernators. New York, NY: Academic Press; 1978.

[84] Zucker I. Circannual clocks: circannual rrhythms: mammals. New York, NY: Plenum Publishers; 2001.

[85] Concannon P, Roberts P, Baldwin B, Tennant B. Long-term entrainment of circannual reproductive and metabolic cycles by northern and southern hemisphere photoperiods in woodchucks (*Marmota monax*). Bio Reprod 1997; (57):1008-1015.

[86] Kondo N, Sekijima T, Kondo J, Takamatsu N, Tohya K, Ohtsu T. Circannual control of hibernation by HP complex in the brain. Cell 2006; (125):161-172.

[87] Monecke S, Saboureau M, Malan A, Bonn D, Masson-Pevet M, Pevet P. Circannual phase-response curves to short and long photoperiod in the European hamster. J Biol Rhyth 2009; (24):413-426.

[88] Ducker MJ, Bowman JC, Temple A. The effect of constant photoperiod on the expression of oestrous in the ewe. J Reprod Fertil 1973; (Suppl 19):143-150.

[89] Gomez-Brunet A, Santiago-Moreno J, del Campo A, Malpaux B, Chemineau P, Tortonese DJ, Gonzalez-Bulnes A, Lopez-Sebastian A. Endogenous circannual cycles of ovarian activity and changes in prolactin and melatonin secretion in wild and domestic female sheep maintained under a long-day photoperiod. Biol Reprod 2008; (78):552-562.

[90] Gwinner E. Circannual rhythms: endogenous annual clocks in the organization of seasonal processes. Berlin: Springer-Verlag; 1986.

[91] Gwinner E. Circadian and circannual programmes in avian migration. J Exp Biol 1996; (199):39-48.

[92] Piersma T, Brugge M, Spaans B, Battley PF. Endogenous circannual rhythmicity in body mass, molt and plumage of great knots (*Calidris tenuirostris*). The Auk 2008; (125):140-148.

[93] Wikelski M, Martin LB, Scheuerlein A, Robinson MT, Robinson ND, Helm B, Hau M, Gwinner E. Avian circannual clocks: adaptive significance and possible involvement of energy turnover in their proximate control. Phil Trans Roy Soc B Biol Sci 2008; (363):411-423.

[94] Carmichael MS, Zucker I. Circannual rhythms of ground squirrels: a test of frequency demultiplication hypothesis. J Biol Rhythms 1986; (1):277-284.

[95] Nisimura J, Numata H. Evaluation of the frequency demultiplication hypothesis of circannual pupation rhythm in the varied carpet beetle *Anthremus verbasci* (Coleoptera: Dermestidae). Biol Rhythm Res 2002; (33):255-260.

[96] Hazlerigg DG, Andersson H, Johnston JD, Lincoln G. Molecular characterization of the long-day response in the Soay sheep, a seasonal mammal. Curr Biol 2004; (14):334-339.

[97] Nakao N, et al. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 2008; (452):317-322.

[98] Woodfill CJ, Wayne NL, Moenter SM, Karsch FJ. Photoperiodic synchronization of a circannual reproductive rhythm in sheep: identification of season-specific time cues. Biol Reprod 1994; (50):965-976.

[99] Lincoln GA. Decoding the nightly melatonin signal through circadian clockwork. Mol Cell Endocrinol 2006b; (252):69-73.

[100] Hazlerigg DG, Morgan PJ, Messager S. Decoding photoperiodic time and melatonin in mammals: what can we learn from the pars tuberalis? J Biol Rhythms 2001; (16):326-335.

[101] Lincoln GA, Andersson H, Hazlerigg D. Clock genes and the long-term regulation of prolactin secretion: evidence for a photoperiod/circannual timer in pars tuberalis. J Neuroendocrinol 2003a; (15):390-397.

[102] Bockmann J, Bockers TM, Winter C, Wittkowski W, Winterhoff H, Deufel T, Kreutz MR. Thyrotropin expression in hypophyseal pars tuberalis-specific cells is 3,5,3'-triiodothyronine, thyrotropin-releasing hormone, and pit-1 independent. Endocrinology 1997; (138):1019-1928.

[103] Ono H, Nakao N, Yoshimura T. Identification of the photoperiodic signaling pathway regulating seasonal reproduction using the functional genomics approach. Gen Comp Endocrinol 2009; (163):2-6.

[104] Goldman BD. Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J. Biol. Rhythms 2001; (16):283-301.

[105] Lincoln GA, Johnston JD, Andersson H, Wagner G, Hazlerigg DG. Photorefractoriness in mammals: dissociating a seasonal timer from the circadian-based photoperiod response. Endocrinology 2005; (146):3782-3790.
[106] Lincoln GA, Libre EA, Merriam GR. Long-term reproductive cycles in rams after pinealectomy or superior cervical ganglioectomy. J Reprod Fertil 1989; (85):687-704.

[107] Gwinner E. Circannual rhythms in birds. Curr Opin Neurobiol 2003; (13):770-778.

[108] Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature 2002; (418):935-941.

[109] Kalra SP, Kalra PS. Brain endocrinology: steroid-peptide interaction in the endocrine brain: Reproduction. New York, NY: Raven Press, Ltd; 1991.

[110] Kalra SP, Allen LG, Sahu A, Kalra PS. Brain opioid systems in reproduction: opioids in the steroid-adrenergic circuit regulating LH secretion: Dynamics and Diversities. Oxford, UK: Oxford University Press; 1989.

[111] Kalra SP, Allen LG, Sahu A, Kalra PS, Crowley WR. Brain-gut peptides and reproductive function: opioids and induction to ovulation: mediation by neuropeptide Y. Boca Raton, FL: CRC press; 1991.

[112] Simerly RB, Gorski RA, Swanson LW. Neurotransmitter specificity of cells and fibers in the medial preoptic nucleus: an immunohistochemical study in the rat. J Comp Neurol 1986; (246):343-363.

[113] Simerly RB, McCall LD, Watson SJ. Distribution of opioid peptides in the preoptic region. Immunohistochemical evidence for a steroid-sensitive encephalin sexual dimorphism. J Comp Neurol 1988; (276):442-459.

[114] Simerly RB, Swanson LW. The distribution of neurotransmitter-specific cells and fibers in the anteroventral perivenrtricular nucleus: implications of control of gonadotropin secretion in the rat. Brain Res 1987; (400):11-34.

[115] Simerly RB. Prodynorphin and proenkephalin gene expression in the anteroventral periventricular nucleus of the rat: sexual differentiation and hormonal regulation. Mol Cell Neurosci 1991; (2):473-484.

[116] Meister B, Ceccatelli S, Hokfelt T, Anden NE, Anden M, Theodorsson E. Neurotransmitters, neuropeptides and binding sites in the rat mediobasal hypothalamus: effects of monosodium glutamate (MSG) lesions. Exp Brain Res 1989; (76):343-368.

[117] Leaden CA, Kalra SP. The effects of endogenous opioid peptides and opiates on luteinizing hormone and prolactin secretion in ovariectomized rats. Neuroendo 1985; (41):342-352.

[118] Kalra SP, Gallo RV. Effects of intraventricular administration of catecholamines on luteinizing hormone release in morphine-treated rats. Endocrinology 1983; (113):23-28.

[119] Schillo KK, Kuehl D, Jackson GL. Do endogenous opioid peptides mediate the effects of photoperiod on release of luteinizing hormone and prolactin in ovariectomized ewes? Biology of Reproduction 1985; (32):779-787.

[120] Malven PV, Stanisiewski EP, and Hagloff SA. Ovine brain areas sensitive to naloxone-induced stimulation of luteinizing hormone release. Neuroendocrinology 1990; (52):373-381.

[121] Aurich C, Schlote S, Hoppen H-O, Klug E, Hoppe H, Aurich JE. Effects of the opioid antagonist naloxone on release of luteinizing hormone in mares during the anovulatory season. Endocrinology 1994; (142):139-144.

[122] Behrens C, Aurich JE, Klug E, Nadmann H, and Hoppen H-O. Inhibition of gonadotrophin release in mares during the luteal phase of the oestrous cycle by endogenous opioids. Journal of Endocrinology 1984; (98):509-514.

[123] Quigley ME, Yen SSC. The role of endogenous opiates on LH secretion during the menstrual cycle. Journal of Clinical Endocrinology 1980; (51):179-181.

[124] Brooks AN, Lamming GE, Lees PD, Haynes NB. Opioid modulation of LH secretion in the ewe. Journal of Reproduction and Fertility 1986; (76):693-708.

[125] Currie WD, Rawlings N. Naloxone enhances LH but not FSH release during various phases of the estrous cycle. Late Sciences 1987; (41):1207-1214.

[126] Malven PV, Hudgens RE. Naloxone-reversible inhibition of luteinizing hormone in postpartum ewes. Journal of Animal Science 1987; (65):196-202.

[127] Barb CR, Kraehng RR, Rampacek GB, and Whisnant CS. Influence of the stage of the estrous cycle on endogenous opioid modulation of luteinizing hormone, prolactin, and cortisol secretion. Biology of Reproduction 1986; (35):1162-1167.

[128] Irvine CHG and Alexander S. Secretory patterns and rates of gonadotropin-releasing hormone revealed by intensive sampling of pituitary venous blood in the luteal phase mare. Endocrinology 1993; (132):212-218.

[129] Meyer SL, Goodman RL. Neurotransmitters involved in mediating the steroid-dependent suppression of pulsatile luteinizing hormone secretion in anestrous ewes. Endocrinology 1985; (116):2054-2061.

[130] Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. Proc Natl Acad Sci USA 2005; (102):3052-3057.

[131] Osugi T, Ukena K, Bentley GE, O'Brien S, Moore IT, Wingfield JC, TsuTsui K. Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*): cDNA identification, transcript localization and functional effects in laboratory and field experiments. Journal of Endocrinology 2004; (182):33-42.

[132] Tsutsui K, Ubuka T, Yin H, Osugi T, Ukena K, Bentley GE, Ciccone N, Inoue K, Chowdhury VS, Sharp PJ, Wingfield JC. Mode of action and functional significance of avian gonadotropin-inhibitory hormone (GnIH): a review. J. Exp. Zool. A. Comp. Exp. Biol. 2006; (305):801-806.

[133] Bentley GE, Ubuka T, McGuire NL, Chowdhury VS, Morita Y, Yano T, Hasunuma I, Binns M, Wingfield JC, Tsutsui K. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. General and Compared Endocrinology 2008; (156):34-43.

[134] Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R. Identification and characterization of a gonadotropin- inhibitory system in the brains of mammals. Proc Natl Acad Sci USA 2006; (103):2410-2415.

[135] Smith JT, Coolen LM, Kriegsfeld LJ. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 2008; (149):5770-5782.

[136] Tsutsui K. A new key neurohormone controlling reproduction, gonadotropin-inhibitory hormone (GnIH): Biosynthesis, mode of action and functional significance. Progress in Neurobiology 2009; (88):76-88.

[137] Murakami M, Matsuzaki T, Iwasa T, Yasui T, Irahara M, Osugi T, Tsutsui K. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. Journal of Endocrinology 2008; (199):105-112.

[138] Yin H, Ukena K, Ubuka T, Tsutsui K. A novel G proteincoupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity. J Endocrinol. 2005; (184):257-266.

[139] Bentley GE, Perfito N, Ukena K, Tsutsui K, Wingfield JC. Gonadotropin-inhibitory peptide in song sparrows (Melospiza melodia) in different reproductive conditions, and in house sparrows (Passer domesticus) relative to chicken-gonadotropin-releasing hormone. J Neuroendocrinol. 2003; (15):794-802.

[140] Ducret E, Anderson GM, Herbison AE. RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. Endocrinology 2009; (150):2799-2804.

[141] Izumi S, Stojilkovic SS, Catt KJ. Calcium mobilization and influx during the biphasic cytosolic calcium and secretory responses in agonist-stimulated pituitary gonadotrophs. Arch Biochem Biophys. 1989; (275):410-428.

[142] Stojilkovic SS, Catt KJ. Calcium oscillations in anterior pituitary cells. Endocr Rev. 1992; (13):256-280.

[143] Leong DA. A model for intracellular calcium signaling and the coordinate regulation of hormone biosynthesis, receptors and secretion. Cell Calcium 1991; (12):255-268.

[144] Rizwan MZ, Porteous R, Herbison AE, Anderson GM. Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. Endocrinology 2009; (150):1413-1420.

[145] Bentley GE, Jensen JP, Kaur GJ, Wacker DW, Tsutsui K, Wingfield JC. Rapid inhibition of sexual behavior by gonadotropin-inhibitory hormone (GnIH). Hormones and Behavior 2006; (49):550-555.

[146] Ciccone NA, Dunn IC, Boswell T, Tsutsui K, Ubuka T, Ukena K, Sharp PJ. Gonadotrophin inhibitory hormone depresses gonadotrophin α and follicle- stimulating hormone β subunit expression in the pituitary of the domestic chicken. J Neuroendocrinol. 2004; (16):999-1006.

[147] Johnson MA, Fraley GS. Rat RFRP-3 alters hypothalamic GHRH expression and growth hormone secretion but does not affect KiSS-1 gene expression or the onset of puberty in male rats. Neuroendocrinology 2008; (88):305-315.

[148] Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 2004; (145):4073-4077.

[149] Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion inhibits LH secretion and has variable effects on sex behavior in the adult male rat. Horm. Behav. 2007; (51):171-180.

[150] Kadokawa H, Matsui M, Hayashi K, Matsunaga N, Kawashima C, Shimizu T, Kida K, Miyamoto A. Peripheral administration of kisspeptin-10 increases plasma concentrations of growth hormone as well as luteinizing hormone in prepubertal Holstein heifers. Journal of Endocrinology in Press, 2008.

[151] Samson WK, Keown C, Samson CK. Prolactin-releasing peptide and its homolog RFRP-1 act in hypothalamus but not in anterior pituitary gland to stimulate stress hormone secretion. Endocrine 2003; (20):59-66.

[152] Taylor MM, Samson WK. The prolactin releasing peptides: RF-amide peptides. CMLS, Cell. Mol. Life Sci. 2001; (58):1206-1215.

[153] Takayasu S, Sakurai T, Iwasaki S, Teranishi H, Yamanaka A, Williams SC, Iguchi H, Kawasawa YI, Ikeda Y, Sakakibara I, Ohno K, Ioka RX, Murakami S, Dohmae N, Xie J, Suda T, Motoike T, Ohuchi T, Yanagisawa M, Sakai J. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. Doi: 10.1073/pnas.0602371103, Published online before print, 2006.

[154] Dockray GJ. The Expanding Family of RFamide Peptides and They Effects on Feed Behaviour. The Physiological Society 2004; OI:10.1113/expphysiol.2004.027169.

[155] Tachibana T, Sato M, Takahashi H, Ukena K, Tsuitsui K, Furuse M. Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. Brain Res. 2005; (1050):94-100.

[156] Bronson FH. Mammalian reproductive biology. Chicago: University of Chicago Press; 1989.

[157] Wilson FE. Neither retinal nor pineal photoreceptors mediate photoperiodic control of seasonal reproduction in American tree sparrows (*Spizella arborea*). J Exp Zool. 1991; (259):117-127.

[158] Juss TS, Meddle SL, Servant RS, King VM. Melatonin and photoperiodic time measurement in Japanese quail (*Coturnix coturnix japonica*). Proc Biol Sci. 1993; (254):21-28.

[159] Ohta M, Kadota C, Konishi H. A role of melatonin in the initial stage of photoperiodism in the Japanese quail. Biol Reprod. 1989; (40):935-941.

[160] Rozenboim I, Aharony T, Yahav S. The effect of melatonin administration on circulating plasma luteinizing hormone concentration in castrated White Leghorn roosters. Poult Sci. 2002; (81):1354-1359.

[161] Barker-Gibb ML, Clarke IJ. Effect of season on neuropeptide Y and galanin within the hypothalamus of the ewe in relation to plasma luteinizing hormone concentrations and the breeding season: an immunohistochemical analysis. J Neuroendocrinol 2000; (12):618-626.

[162] Barrell GK, Moenter SM, Caraty A, Karsch FJ. Seasonal changes of gonadotropin-releasing hormone secretion in the ewe. Biol Reprod 1992; (46):1130-1135

[163] Robinson JE, Radford HM, Karsch FJ. Seasonal changes in pulsatile luteinizing hormone (LH) secretion in the ewe: relationship of frequency of LH pulses to day length and response to estradiol negative feedback. Biol Reprod 1985; (33):324-334.

[164] Revel FG, Saboureau M, Pevet P, Simonneaux V, Mikkelsen JD. RFamide- related peptide gene is a melatonin-driven photoperiodic gene. Endocrinology 2008; (149):902-912.

[165] Sharp DC, Vernon MW, Zary MT. Alteration of seasonal reproductive patterns in mares following superior cervical ganglionectomy. J Reprod Fertil Suppl. 1979; (27):87-93.

[166] Turner JE, Irvine CHG. The effect of various gonadotropinreleasing hormone regimens on gonadotrophins, follicular growth and ovulation in deeply anestrous mares. J Reprod Fertil Suppl 1991; (44):213-55.

[167] Silvia PJ, Meyer SL, and Fitzgerald BP. Pulsatile gonadotropin secretion determined by frequent sampling from the intercavernous sinus of the mare: possible modulatory role of progesterone during luteolysis. Biology of Reproduction 1995; (53):438-446.

[168] Adams TE, Horton MB, Watson JG, Adams BM. Biological activity of luteinizing hormone (LH) during the estrous cycle of mares. Domest Anim Endocrinol. 1986; (93):69-77.

[169] Matteri RL, Papkoff H. Characterization of equine luteinizing hormone by chromatofocusing. Biol. Reprod. 1987; (36):261-269.

[170] Shand N, Alexander SL, Irvine CH. Comparison of the microheterogeneity of horse LH and FSH in the pituitary gland with that secreted intro pituitary venous blood at oestrus. J. Reprod. Fertil. Suppl. 1991; (44):1-11.

[171]. Heidorn RS, Barb CR, Rogers CJ, Hausman GJ, Lents CA. Effects of RFamide-related peptide-3(RFRP-3) on secretion of LH in ovariectomized prepubertal gilts. Journal of Animal Science 2010; (88E (Suppl.2)):566.

[172]. Johnson AL.Gonadotropin-releasing hormone treatment induces follicular growth and ovulation in seasonally anestrous mares. Biol Reprod. 1987;36(5):1199-206.

[173]. Becker SE, Johnson AL. Effects of gonadotropin-releasing hormone infused in a pulsatile or continuous fashion on serum gonadotropin concentrations and ovulation in the mare. J Anim Sci. 1992;70(4):1208-15.

[174]. Thorson JF, Allen CC, Amstalden M, and Williams GL. GnRH therapeutics in the anovulatory mare revisited: physiological implications and clinical benefits of inducing reproductive transition with native hormone. Animal Reproduction Science (Supplement) 2010; 121(1-2):22-23.

APPENDIX

PROCEDURES

Equine LH RIA

References:

Davis SL, Riechert LE, Niswender GD. Biol. Reprod. 4:415- (1971)

Echternkamp SE, Bolt DJ, Hawk HW. J. Anim. Sci. 42:893- (1976)

Golter TD, Reeves JJ, O'Mary CC, Arimura A, Schally AV. J. Anim. Sci. 37:123- (1973)

Niswender GD, Riechert LE, Midgley AR, Nalbandov AV. Endocrinology 84:1166- (1969)

Williams GL, Ray DE. J. Anim. Sci. 50:906- (1980)

1. Iodinated Product: Iodination grade eLH (AFP-5130A).

2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:125,000.

3. Standards: Iodination grade eLH (AFP-5130A). Range 0.1 – 20.0 ng/ml.

4. References: eLH added to equine serum

5. RIA Procedure:

    A. Day 1: Begin Assay

        1. NSB – 500 µl of 1% PBS-EW (egg white).

        2. 0 Std – 500 µl of 1% PBS-EW

        3. Stds – 200 µl std + 300 µl of 1% PBS-EW.

        4. Ref – 200 µl ref + 300 µl of 1% PBS-EW.

        5. Unknown – 200 µl sample + 300 µl of 1% PBS-EW.

6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only.

7. Pipette 200 µl of anti-eLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes.

8. Pipette 100 µl 125I-eLH (20,000 cpm/100 µl diluted in 1% PBS-EW) to all tubes.

9. Vortex tubes briefly and incubate for 24 h at 4°C.

B. Day 2: Add Second Antibody

1. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes.

2. Vortex tubes briefly and incubate for 48-72 h at 4°C.

C. Day 4: Pour Off Assay

1. Add 3 ml ice cold PBS (0.01 M; pH 7.0) to all test tubes except TC tubes.

2. Centrifuge tubes for 1 h at 4°C at 3600 rpm.

3. Decant supernatant.

4. Count radioactivity of each tube using a gamma counter

Progesterone RIA

Single Antibody RIA Kit, Diagnostic Products Corporation, Los Angeles, CA

References:

Jones EJ, Armstrong JD, Harvey RW. J. Anim. Sci. 69:1607 – (1991)

Diagnostic Products Corporation Coat-A-Count Progesterone Kit, Los Angeles, CA

Simpson, R.B., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 70: 1478– (1992).

1. Iodinated Product: Iodination grade hP4.

2. Antibody: Anti-human P4 coated tubes.

3. Standards: Human serum with added P4. Range 0.1 – 20.0 ng/ml.

4. Reference: Human standard preparation added to bovine serum.

5. RIA Procedure:

A. Begin and complete assay

1. Pipette in non-coated polypropylene tubes

NSB – 100 µl of 0 Std

2. Pipette in antibody coated tubes

0 Std – 100 µl

Std – 100 µl

Ref – 100 µl

Unknowns – 100 µl

3. Pipette 1 ml of 125I –P4 provided in the kit to all tubes including two Total Count non-coated polypropylene tubes.

4. Vortex tubes briefly and incubate at room temperature for 3 h.

5. Pour off supernatant.

6. Count radioactivity of each tube using a gamma counter

VITA

Ligia Dias Prezotto

Address:

NORTH DAKOTA STATE UNIVERSITY, Hultz Hall, 108, Dept. 7630, PO Box 6050, Fargo, ND, 58108-6050, phone: 701-231-7630, mobile phone: 979-571-8920
e-mail: ligiaprezotto@hotmail.com

Education:

B.S, Animal Science, Faculdade de Zootecnia e Engenharia de Alimentos-University of Sao Paulo, Pirassununga-SP-Brazil

M.S., Physiology of Reproduction, Texas A&M University, College Station, TX 2012

Selected Publications:

Prezotto LD, Thorson JF, Cardoso RC, Amstalden M, Williams GL. Failure of an equine homologue of avian gonadotropin inhibiting hormone to alter secretion of luteinizing hormone in the mare. Biol. Repro Special Issue 2011; 85:6.

Thorson JF, Prezotto LD, Cardoso R, Alves BRC, Liu S, Edwards J, Amstalden M, Williams GL. Effects of RF-amide related peptide 3 (RFRP-3), an equine homolog of avian gonadotropin-inhibiting hormone, on adenohypophyseal responsiveness to GnRH in mares. Biol. Repro Special Issue 2011; 85:6.

Williams GL, Thorson JF, Prezotto LD, Velez IC, Cardoso RC, Amstalden M. Reproductive seasonality in the mare: neuroendocrine basis and pharmacological control. Dom Animal Endo. 2012; 42.

Thorson JF, Prezotto LD, Cardoso RD, Alves BRC, Amstalden M, Williams GL. GnRH therapeutics to advance the timing of pregnancy in the seasonally anovulatory mare. J. Anim. Sci. Suppl 2011; 89:685.

Williams G, Stanko R, Allen C, Cardoso R, Prezotto L, Thorson J, Amstalden M. Evidence that prostaglandin administration at the onset of a 5-day CO-Synch + CIDR synchronization protocol markedly improves fixed-time AI pregnancy rates in Bos indicus-influenced cattle. J. Anim. Sci. Suppl 2011; 100:264.